Volume XXVIII October, 1929 Number 4

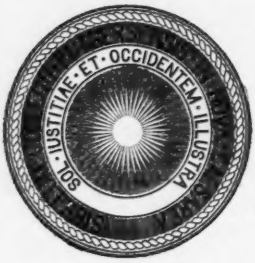
SOIL SCIENCE

Contents

PUBLISHED MONTHLY
THE WILLIAMS & WILKINS COMPANY
MT. ROYAL AND GUILFORD AVENUES
Baltimore, Maryland, U. S. A.

Made in United States of America

SOIL SCIENCE



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EDITORIAL BOARD

Entered as second-class matter May 12, 1919, at the post office at Baltimore, Maryland, under the act of March 3, 1879

RELATION OF CALCIUM TO THE NODULATION OF SOYBEANS ON ACID AND NEUTRAL SOILS

W. A. ALBRECHT AND FRANKLIN L. DAVIS[1]

University of Missouri

Received for publication April 20, 1929

As a result of recent tests on the effects of calcium-bearing soil treatments, such as lime, acid phosphate, and calcium salts, upon nodulation of soybeans, there is an indication that the beneficial effect of these materials is essentially one of calcium stimulation. Frequent and repeated reports of failures to obtain nodulation of legumes by the pure culture method on certain acid soil types of northeastern Missouri and southeastern Illinois led to a search for the responsible factors. Soybeans, as an acid tolerant legume, have taken a prominent place in this territory within recent years, but thorough inoculation of even this crop has been difficult unless the soil was well limed. A study of the beneficial effects of lime on soybean inoculation, reported herein, leads to the belief that much of this is due to the element calcium.

HISTORICAL

Harper and Murphy (7), have recently given a review of the literature dealing with the factors affecting nodule formation by soybean plants. Other recent papers summarize the literature similarly, including specific phases of inoculation. Wilson (14). Scanlan (12), and Karraker (9), have each reported an increase in nodulation in consequence of the application of calcium as the carbonate or as a soluble salt. Alway (1), who was comparing the effectiveness of inoculation of alfalfa by soil transfer with that by pure cultures on lime-deficient sandy soils, found these two methods of equal efficacy when the land had been limed well in advance of seeding. But when the land had not been limed, the soil transfer method was far more effective. An increase in the amount of culture, many times beyond the usual rate, did not make it as effective as soil transfer. This seems to indicate an adaptation of the organism to a lime-deficient soil habitat in consequence of several previously grown, host crops. Bryan (4), in a study of the effect of acid soil reactions on nodulation of soybeans, found that, in general, the hydrogen-ion relations for the organisms tend to be the same as those for the host plant. He secured a

[1] Associate professor of soils, college of agriculture, University of Missouri; and assistant professor of soils, North Carolina State College of Agriculture and Engineering, formerly assistant in soils, University of Missouri.

maximum nodulation at pH 6.5 and none below 4.9, although the critical hydrogen-ion concentration for the organisms was found to be pH 3.5 to 3.9. Scanlan (12) concluded that hydrogen-ion concentration must have no direct effect upon inoculation of soybeans by *B. radicicola*. Although both calcium carbonate and calcium acetate stimulated inoculation tremendously, the carbonate neutralized the hydrogen-ion concentration and the acetate had no effect upon it. Fellers (6) noted that the bacterial infection of roots did not take place readily on acid soils even when a good supply of bacteria was present.

Karraker (9), working with alfalfa, single plants of which he grew with part of the roots in a limed and part in a lime-deficient, acid soil, found that there was a difference in the nodule formation of the two parts of the root system, and that this difference was as great as the difference between the nodulation of plants grown wholly on the limed and those on the lime-deficient soils. He concluded that the effect of soil reaction upon nodule formation must be one of localized character in the plant, a direct effect of soil hydrogen-ion concentration on the bacteria in the nodules, or an antecedent effect of the soil on the bacteria while they are existing non-symbiotically in the soil.

It has been pointed out by Lohnis and Smith (10) that bacteria undergo a fairly definite cyclic change. Bewley and Hutchinson (2) found similar cyclic changes and that these are even specific with reference to cultural conditions. They stated that as long as there was sufficient available carbohydrate to support growth, the organism remained in the motile rod form with no changes. Their "pre-swarmers" (one of their cyclic forms), could be induced by the addition of calcium or magnesium carbonate to the medium. Of a considerable number of compounds other than carbohydrates, calcium phosphate alone was capable of bringing about the change from "pre-swarmers" to rods. The response to the reaction of the soil, in the main, was a rapid change from the normal rod form to "pre-swarmers" in calcareous soils; a production of a highly vacuolated form and the eventual death of the organisms in acid soil; and a continuous growth without significant change of form in a slightly alkaline soil. Thornton and Gangulee (13), in an even more recent investigation along this line, also noted a similar, regular cycle of change, in which unbanded rods, cocci, and banded rods successively followed each other. They stated that an increase in the percentage of cocci was associated with increased bacterial numbers and with the appearance of motile forms. They found that by modifying the liquid used to suspend the inoculum added to the soil, the time of appearance of cocci in predominance could be altered, and on this basis they recommend the inoculation of the soil with a bacterial suspension in milk to which is added 0.1 per cent of calcium acid phosphate.

That calcium may play some significant part in establishing legume bacteria in certain lime-deficient soils was suggested by previous workers and served as the main hypothesis in the work reported herein.

EXPERIMENTAL

Part I

Increased nodulation of soybeans, upon an already well-inoculated soil, resulted from the use of calcium treatment on such soil in the greenhouse. This was an acid soil (pH 5.5), taken from the experimental field, and already well inhabited with the symbiotic organism in consequence of inoculated crops of soybeans of the three preceding seasons. Limestone, equivalent to 4 tons per 2 million pounds, was added to part of the soil. The seed from a single mother plant was used for a stand of 5 plants per pot on 30 pots from both the limed and the unlimed soil. They were grown for 5 weeks, after which the uniform and healthy plants were taken up and the nodules counted. Although Erdman (5), has presented an argument for the importance of the size as well as the number of nodules in determining the effectiveness of inoculation, the numbers only were taken. The data are summarized in the first half of table 1.

The results show an increase of 336 per cent in numbers of nodules formed as a result of liming, even though the soil was already well inoculated with the organism. In spite of the fact that these data show a correlation between inoculation and neutralization of the soil acidity, this does not necessarily establish a causal relationship.

Part II

In consequence of the fact that an application of calcium as the carbonate, produced an important increase of nodulation on an already well-inoculated soil, and of the belief that this stimulation was not necessarily the result of a change in hydrogen-ion concentration, or at least not entirely so, a test was made of the effect of calcium, as the chloride, upon the inoculation of soybeans by the pure culture method on an acid soil which was sterile with regard to *B. radicicola* of soybeans. To each of 30 pots of a rather heavy Union silt loam (pH 5.4), there were added at planting time 25 cc. of a solution of calcium chloride supplying calcium equivalent to that of 200 pounds of calcium carbonate per 2 million of soil. Thirty pots of the untreated soil were planted also. Liberal quantities of an inoculating suspension were supplied directly to the beans at the time of planting. Eight pots of untreated and uninoculated soil were planted as checks. When, at the end of 5 weeks, the examination for nodules revealed none formed, 16 pots each of the inoculated, calcium treated and of the inoculated, untreated soil were immediately replanted with sprouted beans in the same pots. After 5 weeks of growth, these were likewise carefully taken up, washed, and examined for nodules. The complete data are summarized in the latter half of table 1.

The nodulation of the beans of the second planting indicates that the calcium as a chloride has a stimulating effect upon the longevity and viability of the organism in the acid soil. This agrees with Scanlan's results of increased viability of the organism in water cultures.

Part III

Since the addition of a small amount of a neutral calcium salt to an acid soil (pH 5.4) had kept *B. Radicicola* viable within the soil from the time it was applied by pure cultures on the first crop until the second planting, and since an addition of large amounts of calcium carbonate produced important increases in nodulation of soybeans on an already well-inoculated soil, it was thought possible to determine whether this stimulating effect was due, (*a*) to calcium within the plant, (*b*) to an effect of calcium upon the organism in the soil, or (*c*) to an effect of calcium upon the soil as the habitat of the organisms, by growing plants so arranged that one part of the root system

TABLE 1

Nodulation of soybeans on acid soils as influenced by calcium compounds

SOIL TREATMENT	NUMBER OF POTS	TOTAL NUMBER OF PLANTS	RANGE IN NODULES PER POT	AVERAGE NODULES PER PLANT	PER CENT INCREASE
Soil already inoculated given calcium carbonate					
None	30	130	10–81*	12.0	
Calcium carbonate—4 tons	30	133	64–247†	40.2	336
Sterile soil given calcium chloride with inoculation					
First crop None	30	130	—	—	
First crop Calcium chloride	30	133	—	—‡	
Second crop None	16	72	—	—‡	
Second crop Calcium chloride	16	80	1–26	1.8	

* Three pots exceeded this range greatly, having 110, 140, and 142 nodules.

† Two pots exceeded this range greatly, having 297 and 380 nodules.

‡ Three plants had one nodule each in these trials.

TABLE 2

Nodulation of soybeans grown with part of root system in calcium-treated soil and part in untreated soil

TREATMENT	PLANTS WITH DIVIDED ROOTS			CHECK PLANTS			
		Nodule production		Calcium-treated soil		Untreated soil	
	Number of plants	Roots in calcium-treated soil	Roots in untreated soil	Number of plants	Number of nodules	Number of plants	Number of nodules
Uninoculated	4	0	0	6	0	5	0
Inoculated	23	160	77	28	494	31	302
Average per plant		6.95	3.34		17.64		9.74
Per cent increase through calcium		208			181		

of each was growing in an acid soil and the other part in the calcium-treated soil.

Soybean seedlings were grown in sterile sand from 10 to 14 days, or until the lateral roots about an inch long were sufficient to support the plant. These seedlings were taken up, washed, and the tap roots cut off just below the longest lateral roots. They were then planted with half of their roots on one side and half on the other side of the water-tight partition of a two-compartment pan. The moist acid soil was filled in around the roots on one side, and moist calcium-treated soil on the other. Liberal quantities of the inoculating suspension were supplied directly to the roots. The soil used was a Putnam silt loam (pH 5.14). Five plants were planted with their roots divided by the partition and five more plants with their tap roots similarly pruned were planted wholly on each side of the partition as checks. Although there was a high mortality of plants, those that lived grew satisfactorily for the five weeks, after which a count of the nodules was made. The results are given in table 2.

The increase in nodulation on the parts of the root systems growing in the calcium-treated soil over those parts growing in the untreated soil was comparable to the increase obtained in the checks or those whose entire root system was within a single soil treatment. This agrees with the results obtained by Karraker (9) on alfalfa. Although this type of experiment is unsatisfactory because of unequal development of the divided parts of the root, the results indicate that the stimulating effect of the calcium upon nodulation was due to an effect upon the bacteria in the soil, or to a physiological effect within the plant. In addition, the effect was local in character, and limited to the roots. The calcium was not translocated to all parts of the plant root system sufficiently to make its influence uniform on the degree of inoculation, at least not within the time limits of this experiment.

Part IV

The preceding results raised the question whether there is an effect upon nodulation by the calcium already within the plant tissues. An attempted answer was undertaken by growing some soybean seedlings in calcium-free and some in calcium-bearing substrates and then transplanting from both into an inoculated soil. The calcium-free substrate was prepared by treating sand with 5*N* hydrochloric acid for 3 hours, washing with water until acid-free according to the silver nitrate test, drying, and sterilizing in an oven at 110°C. for 48 hours. For the calcium-bearing substrate, calcium carbonate was mixed with the same quartz sand at the rate of 10,000 pounds per 2 million. This was also sterilized in the oven 110°.

The total yield of beans from a single plant was sterilized, germinated for 24 hours, and planted into pots of these sterile substrates, half into the calcium-free and half into the calcium-bearing sand. The pots were set to their shoulders into moist soil, which was sterile with regard to *B. radicicola*, and after 10 or 11 days the plants were taken up, washed, and replanted to the

inoculated soil which had been prepared and sifted at a suitable moisture content into ordinary greenhouse flats. Both the calcium-bearing and calcium-starved seedlings were grown simultaneously on their respective halves of the same flat and within the same soil. Seedlings so treated were transplanted and grown on two different soils, one an acid, lime-deficient soil, and the other, a neutral, fertile, garden soil. No inoculation was added, since both soils had grown well-inoculated crops of soybeans during two consecutive seasons just previous to this test. After a growth of 5 weeks, the plants were taken up readily without injury to the roots and the nodules counted. The data from the count are summarized in table 3. Included in the table are also the analytical data giving, (*a*) the calcium content of the soybean seeds from a single, similar plant, (*b*) the calcium content of 10-day-old calcium-bearing and calcium-starved seedlings, and (*c*) the total electrodialyzable calcium of the soils as determined by Bradfield's (3) method of measuring the total electro-

TABLE 3

Nodulation of soybeans in neutral and acid soils as influenced by the calcium in the seedlings

SOIL CHARACTER	pH	SEEDLING TREATMENT	NUMBER OF PLANTS	NODULE NUMBERS PER PLANT		CALCIUM CONTENT		
				Range	Average	Per 100 seedlings	Electrodialyzable per 10 gm. soil	Per 100 seeds
						mgm.	*mgm.*	*mgm.*
Neutral	7.8	None	60	12–77	36.6	17.07	24.07	6.85
		Calcium	67	9–67	38.9	30.14		
Acid	5.5	None	69	1–7	3.4	17.07	11.78	6.85
		Calcium	79	2–25	15.1	30.14		

dialyzable base. These analyses were made in order to correlate the nodulation with the calcium content of the plants as influenced by the treatments.

The increased nodulation of the calcium-bearing seedlings on the calcium-deficient, acid soil demonstrates that the presence of calcium within the plant increases nodulation of soybeans on such soil. On the other hand, the lack of difference in nodulation of the seedlings on the neutral, calcium-laden soil indicates that the presence of this element within the plant on a calcium-sufficient soil does not affect nodulation, or that if it does, the calcium-starved plants are able to take calcium from the soil rapidly enough to offset the measurable differences in nodulation.

The increase in calcium content of the calcium-starved seedlings over that in the seed, as shown by the analytical data, was due to the calcium that was carried back into the acid-extracted sand by the tap water with which it was washed. An elimination of this factor might have served to intensify further the differences obtained.

Part V

Since calcium exerted an intimate effect upon inoculation by the organism *B. radicicola*, this effect was deemed possible through an inter-relation with the soil colloids, the main chemically reactive part of the soil. It is known that the colloids are highly absorptive, and that minute quantities of calcium are effective in flocculating them, hence this phase of the experiment was undertaken to detect such possible relation.

A suspension in distilled water of the organisms from several agar cultures was added to a 0.4 per cent solution of colloidal clay in a ratio of four parts of the bacterial suspension to five parts colloidal solution. To this mixture was added one part of water containing the desired amount of flocculating agent.

TABLE 4

Nodule numbers on soybeans inoculated by colloidal clay suspensions of bacteria

KIND OF INOCULATING SUSPENSION	RANGE IN NUMBER OF NODULES PER POT*	DESCRIPTION OF NODULATION
Distilled water	72–90	Variable size. Scattered over entire root system
Colloidal clay	84–112	Uniform size. Clumped at plant crown
Tap water	56–71	Variable size. Well scattered
No inoculation	0–0	Plants yellow. Grew for time of test
Full inoculation	98–161	Variable size. Well distributed
Calcium chloride supernatant	0–6	Not over one plant per pot infected
Calcium chloride flocculant	64–133	Variable size. Clumped at plant crown
Potassium chloride supernatant	85–169	Variable size. Well distributed
Potassium chloride flocculant	148–162	Indiscriminate size. Clumped at base, some scattered

* Duplicate pots were grown with 5 plants each.

Those mixtures left unflocculated received the equivalent of distilled water. Thus the resulting solutions contained 0.2 per cent colloidal clay and equal numbers of organsms throughout. These were made up in units of 100 cc. in test tubes. The chlorides of potassium and calcium were used as comparative flocculating agents at the rate of 0.5 milliequivalents, or the minimum requirement of potassium chloride as electrolyte at this concentration of colloidal clay. Mixtures of the organism at the same concentrations in the natural colloid, in distilled water, and in tap water were set up as checks.

The tubes were incubated for 7 days, after which the liquid supernatant to the flocculated clay, the flocculated clay itself, the natural colloidal clay, and the suspensions in water, were tested for the presence of the viable organism by applying specific quantities to sterile, germinated soybeans as they were

planted into sterile sand. At the end of 5 weeks the plants were taken up and the nodules per plant counted. The data are presented in table 4. Plate 1 shows clearly the type and extent of nodulation of the roots from the various solutions.

The results obtained in this experiment were duplicated almost identically in a repetition of the experiment 6 weeks later. The nodulation obtained indicated that the colloidal clay absorbed the bacteria but did not destroy their viability. Flocculation of the clay with calcium chloride carried the organisms down and retained them within the flocculant. This was not the case for the potassium chloride. In the potassium chloride the supernatant was as effective for inoculation as the flocculant.

In order to verify the accuracy of this test and to determine whether the calcium chloride or the colloid is the active factor in carrying the organisms out of suspension, this experiment was repeated. Platings were made from the

TABLE 5

Plate counts of B. radicicola as influenced by colloidal clay treatments

PORTION OF TREATMENT SAMPLED	AVERAGE COUNT PER CUBIC CENTIMETER
Supernatant to calcium flocculant	8
Supernatant to potassium flocculant	13,100
Upper half calcium-bacteria suspension	200
Upper half potassium-bacteria suspension	7,000
Lower half calcium-bacteria suspension	4,300
Lower half potassium-bacteria suspension	1,000
Supernatant to centrifuged inoculated colloid	22,100
Inoculated colloid—not centrifuged	9,550,000
Tap water suspension	3,600

solutions into sterile petri dishes at the time of planting. Also, the pure bacterial suspensions were flocculated by potassium chloride and calcium chloride and then plated. A sterile colloidal clay inoculated 7 days previously was also plated. The effectiveness of the absorption of the bacteria by the colloid was tested by centrifuging the colloidal material out of an inoculated colloidal clay and then plating the centrifuged solution. The counts are given in table 5.

The relation of the calcium to the retention of the bacteria by the flocculated clay, as previously found, was substantiated in this trial. The liquid, supernatant to the potassium chloride flocculant contained over 13,000, whereas that over the calcium chloride flocculant contained but 8 bacteria per cubic centimeter, showing that the calcium flocculated clay carried the bacteria out almost completely whereas the potassium flocculated clay did not. Calcium used independently of the clay, carried out the bacteria, since the water suspension given potassium chloride contained a count of 7000, whereas the treatment with calcium chloride reduced this to 200 per cubic centimeter. In

comparing the inoculated colloid suspension with the same after centrifuging, the number of about ten million in the former was reduced to about 22,000 in the supernatant in consequence of centrifuging.

These data suggest that though the clay carries the bacteria out of suspension, certainly the calcium does likewise, whether used alone or whether combined with the clay colloid. When the calcium is used in conjunction with the clay, however, a more nearly complete removal of the organisms is obtained. This is no doubt due to the simultaneous coagulation of the bacteria and to the flocculation of the colloid. This does not hold true for the potassium chloride.

TABLE 6

Nodulation of soybeans on acid soils in field treatments of calcium

INOCULATION TREATMENT	MARION SILT LOAM*					PUTNAM SILT LOAM (BETTER PHASE)†			
			Nodule					Nodule	
	Nodules per plant	Per cent infected plants	Volume, $\frac{\text{cc.}}{1000}$ per nodule	Weight, mgm. per nodule	pH	Nodules per plant	Per cent infected plants	Volume, $\frac{\text{cc.}}{1000}$ per nodule	Weight, mgm. per nodule
None‡	0.2	20.0			6.4	0	0		
Culture	0.6	6.6	900.0	133.0	6.1	3.5	43	9.5	9.2
Culture and calcium chloride	26.6	100.0	24.1	24.4	6.0	3.3	67	25.0	21.2
Culture and calcium nitrate	22.9	100.0	28.6	28.9	6.0	3.6	50	23.3	23.0
Culture and calcium hydroxide	22.9	100.0	21.1	23.0	6.0	8.9	83	33.4	36.8
Inoculated soil	16.0	100.0	31.2	30.0	6.4	2.6	57	40.2	38.8
Soil and calcium chloride§	13.4	100.0	45.5	44.4	6.4	6.6	87	19.7	17.1
Soil and calcium nitrate¶	11.6	100.0	53.5	58.3	6.4	3.7	60	25.4	20.0

* The initial soil contained 0.9785 as total base (cc. normal acid) and 23.3 mgm. as electrodialyzable calcium per 10 gm. soil.

† The initial soil contained 0.7563 as total base (cc. normal acid) and 18.2 mgm. as electrodialyzable calcium per 10 gm. soil and had a pH of 5.6.

‡ About 30 plants were examined in each case. They contained 1.23 per cent nitrogen in the tops and 0.76 per cent in the roots.

§ The plants in this treatment contained 2.39 per cent N in the tops, 2.04 per cent in the roots.

¶ The plants in this treatment contained 2.16 per cent N in the tops, 2.13 per cent in the roots.

Part VI.—Field Trials

After finding that applications of lime may stimulate nodulation on an acid soil already inhabited by the organisms, and that small amounts of calcium in the soil, as well as small amounts within the plant tissues, are important in stimulating nodulation, it seemed quite plausible that liming a soil may exert its influence not wholly as a secondary effect through the correlation of hydrogen-ion concentration, but also in consequence of its content in calcium. Work was done in the field to test whether small amounts of soluble calcium

with no neutralizing capacity would improve nodulation by the pure culture method on acid soils that were difficult to inoculate without liming.

The work was done in coöperation with farmers experiencing difficulty in getting inoculation on unlimed land. The soybeans were inoculated at planting time with a tested strain of the organism, and applications of calcium chloride, calcium nitrate, calcium acid phosphate, and calcium hydroxide were made through fertilizer attachments on the seeding machinery. In addition to these, tests were made using inoculated soil, both with and without calcium salt treatments. The salts, including calcium chloride and calcium nitrate, were

TABLE 7

Nodulation of soybeans on acid soils in field treatments of calcium

INOCULATION TREATMENTS	PUTNAM SILT LOAM (ROLLING PHASE)*				PUTNAM SILT LOAM (FLAT PHASE)†			
	Nodules per plant	Per cent plants infected	Average nodule: Volume, cc./1000	Average nodule: Weight, mgm.	Nodules per plant	Per cent plants infected	Average nodule: Volume, cc./1000	Average nodule: Weight, mgm.
None (a)	0.6	20	105.5	106.7	0	0		
Culture	10.2	100	48.9	51.1	5.6	66	30.1	30.1
Culture and calcium chloride (b)	5.2	90	31.2	31.8	Dialyzable: Base / Calcium			
Culture and calcium nitrate	9.2	93	29.3	29.9	(a) 0.7665	14.5		
Culture and acid phosphate	8.0	100	37.5	38.3	(b) 0.9647	20.6		
Inoculated soil					3.8	83	47.8	50.4
Soil and calcium chloride	Dialyzable: Base / Calcium				2.1	57.1	50.9	52.1
	(a) 0.6597	18.2						
Soil and calcium nitrate	(b) 0.6597	14.5			1.1	33	26.4	28.2
Soil and limestone‡	25	100	26.8	27.7				

* This untreated soil had a pH of 5.75, given limestone it had a pH of 6.3. The electrodialyzable base and calcium were determined on 10 gm. of these soils after the crop was grown. Limestone was applied at the rate of 3 tons per acre.

† The untreated soil had a pH of 5.14.

‡ Limestone applied was equivalent to 5 tons per 2,000,000 pounds soil.

mixed, as a 2 *N* solution, into the dry pulverized soil and the soil was then dried until it would operate through the fertilizer attachment. A determination of the nodulation was made when the beans were at full growth, just shortly before maturity. Samples of the soil were also taken then for hydrogen-ion measurements and for determinations of dialyzable base and calcium. Tables 6 and 7 give summaries of the data on nodulation in these field trials.

The data show that the culture inoculation was successful on the Marion Silt Loam in every case where it was supplemented by applications of small amounts of calcium salts, but failed wherever the calcium was omitted.

These differences were very noticeable in the color of the plants. The significant differences in the crop and inoculation on this soil in consequence of the calcium treatment are shown in plate 2. Soil inoculation was successful on this soil type without added calcium. The inclusion of calcium, however, increased the size of the nodules significantly. The lessened number of nodules per plant, when calcium was added to the inoculating soils, suggests possible death to the organisms by this salt treatment, though this is not significant enough to reduce the percentage of plants inoculated.

On fields other than the Marion Silt Loam, the culture used alone was successful without special treatment. However, in many cases the addition of the calcium, especially the hydroxide, which distributed itself more thoroughly on account of its fineness, gave increased nodules per plant, and increased the percentages of infection. On these fields the color differences were less pronounced than on the Marion Silt Loam, but yet significant differences in growth were evident, as is shown in plate 3.

Determinations of the hydrogen-ion concentration revealed a pH of 6.0 on the Marion Silt Loam where the calcium was beneficial to inoculation, and a much lower figure for the pH where calcium was less effective. Just what relations exist between the hydrogen-ion concentration and the effectiveness of the applied calcium, or between the effectiveness and the electrodialyzable base or calcium, is still a question. The total electrodialyzable base and electrodialyzable calcium content seem to decrease as the calcium additions were less effective.

Part VII

In an attempt to determine the relation of dialyzable base or calcium of the soil to inoculation, the flat phase of the Putnam Silt Loam of the field trials was used in the greenhouse. Seedlings were started for 10 days in calcium-deficient and calcium-laden substrates and then transplanted to this soil given no treatment, given calcium carbonate equivalent to 5 tons per acre, and given calcium chloride at the rate of 1 part per 1500 parts of soil solution, considering the soil at 25 per cent moisture. Thorough inoculation was applied at planting and the plants were grown for 5 weeks, when they were examined for their nodulation. Analyses were made of the seedlings for their calcium content, and of the soil for the total electrolyzable base and calcium. The hydrogen-ion concentration was also determined. The complete data are given in table 8.

Though no statistical manipulation was undertaken to express the reliability of the data in the usual way, it is interesting to note that even though this soil gave no great improvements in its inoculation through calcium applied in the field trials, a significant increase occurred in the nodule numbers when the seedlings carried a liberal calcium supply. This difference was obliterated when the soil was given calcium, either as carbonate or as chloride. No correlation seemed to exist between the electrodialyzable calcium and the nodule numbers per plant. However, it is interesting to note that the insoluble

calcium carbonate was less effective in increasing nodule numbers per plant, than was the soluble calcium chloride or calcium within the seedlings, for the short time of this trial.

Measurements of the electrodialyzable calcium within the soils in this study were not numerous enough to establish whether or not this quantity of the element might serve as a possible indication of the soil's deficiency in calcium with reference to inoculation. Further data of this kind will be necessary to decide the question fully. However, the data thus far suggest that electrodialysis is scarcely a criterion as to whether or not the soil will yield sufficient calcium to guarantee thorough inoculation or whether added calcium might

TABLE 8

Nodule production by soybeans on acid Putnam Silt Loam

SOIL AND TREATMENT	UNTREATED*		ONE PART $CaCO_3$ PER 200 SOIL		ONE PART $CaCl_2$ PER 1,500 SOIL SOLUTION†	
Seedling treatment	None	Calcium	None	Calcium	None	Calcium
Number of plants	73	61	62	79	70	72
Average number of nodules per plant	7.9	12.8	8.0	7.3	11.9	11.0
Range in number of nodules per plant	0–23	1–26	0–19	0–20	0–34	1–41
Number of plants not inoculated	1	0	2	2	1	0
Calcium content of plants at replanting: mgm. Ca per plant	17.07	30.14	17.07	30.14	17.07	30.14
Total electrodialyzable base in soil at end of plant growth: titrable milliequivalents of base	0.78582		2.15754		0.83412	
Total electrodialyzable calcium in soil at end of plant growth: mgm. Ca per 10 gm. H_2O-free soil	12.9		41.6		13.4	
pH of soil at end of plant growth	5.82		7.40		5.25	

* Original pH of soil—5.14.

† Porosity of treated soil—48.7 per cent. Calculations of soil solution were made for 25 per cent moisture.

improve the establishment of the relation between nodule bacteria and their host plant. Under certain conditions, certainly, very small amounts of calcium are beneficial in establishing thorough inoculation and consequently the legume crop itself. This possibility might be inferred from the work of McCool (11) and by the report of Jaeger (8). How small this amount may be in any case is still a question. Attention may need to be given to ionizable calcium or some other forms before soil analysis can contribute a simple chemical answer to this complex question of biological behavior.

SUMMARY AND CONCLUSIONS

1. The study reported herewith suggests that the beneficial effects of liming for establishing thorough inoculation of legumes on acid soils may be due in part to the element calcium as well as to a change in the degree of acidity.

2. The use of calcium carbonate on an acid soil already well inoculated with *B. radicicola* of soybeans, gave decided improvement in the inoculation of this crop.

3. The addition of calcium chloride to an acid soil, sterile to the soybean organism, favored its longevity from the time of introduction, and improved inoculation on the later planting.

4. A part of the root system of soybean growing in calcium-bearing soil had better inoculation than the part of the same root system growing in calcium-deficient soil. This effect was, then, not readily transmitted to roots in environment deficient in calcium but supplied with the necessary organisms.

5. A liberal supply of calcium within 10-day-old soybean seedlings improved their inoculation when they were transplanted into acid soils.

6. The soybean organisms in colloidal clay suspensions were carried down when flocculated with calcium chloride but not significantly when flocculated with potassium chloride.

7. Field trials found very small quantities of calcium, applied as different salts, a very effective help in increasing inoculation on certain soils and scarcely significant on others.

8. The effect of calcium in stimulating inoculation failed to show a significant correlation to the hydrogen-ion concentration, or the electrodialyzable calcium in the soil in the few cases studied.

9. Though thorough inoculation may be stimulated in some cases by the addition of calcium, many factors, as fertility of soil and cultural practices are also of significance.

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PLATE 1

SOYBEAN NODULES BY INOCULATION WITH BACTERIA IN COLLOIDAL CLAY

FIG. 1. Distilled water suspension.
FIG. 2. Calcium chloride supernatant.
FIG. 3. Calcium chloride flocculant.
FIG. 4. Colloidal clay suspension.
FIG. 5. Potassium chloride supernatant.
FIG. 6. Potassium chloride flocculant.

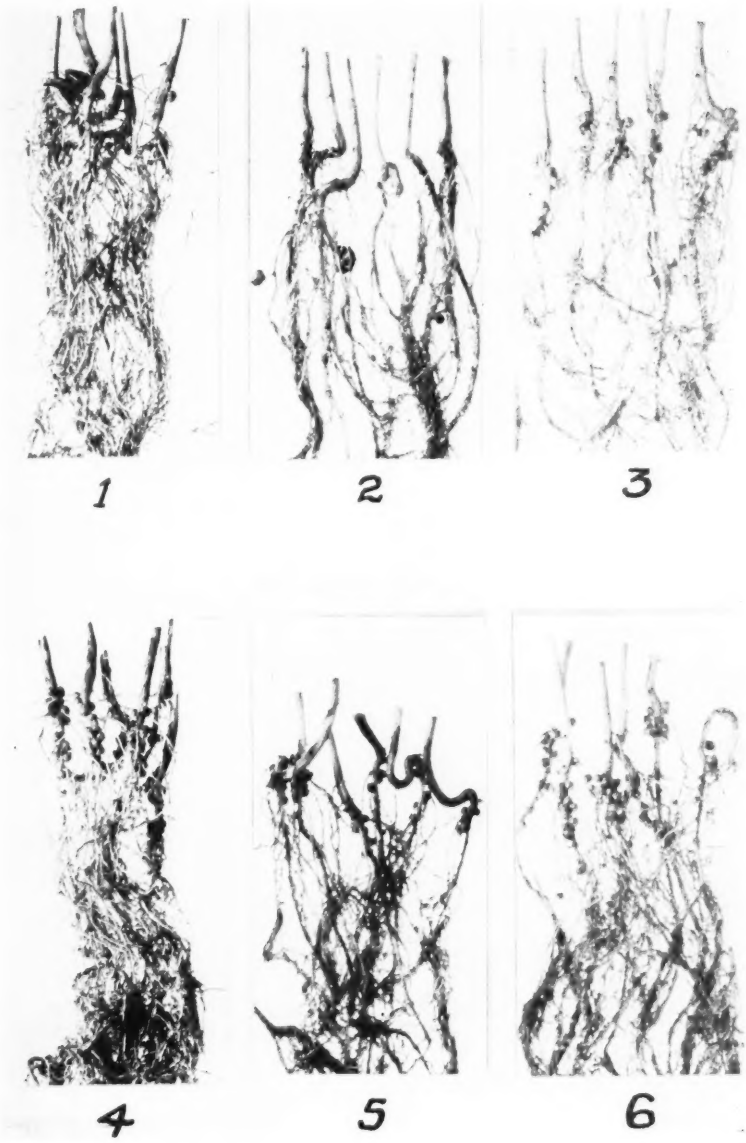

PLATE 2

DIFFERENCE IN COLORATION AND NODULATION OF SOYBEANS DUE TO CALCIUM TREATMENT

FIG. 1. Differences in color in consequence of calcium treatment (Marion Silt Loam). Left, cultures and calcium chloride. Right, cultures only.

FIG. 2. Nodulation differences in consequence of calcium treatment. (Marion Silt Loam.) Above, cultures and calcium. Below, cultures only.

FIG. 1

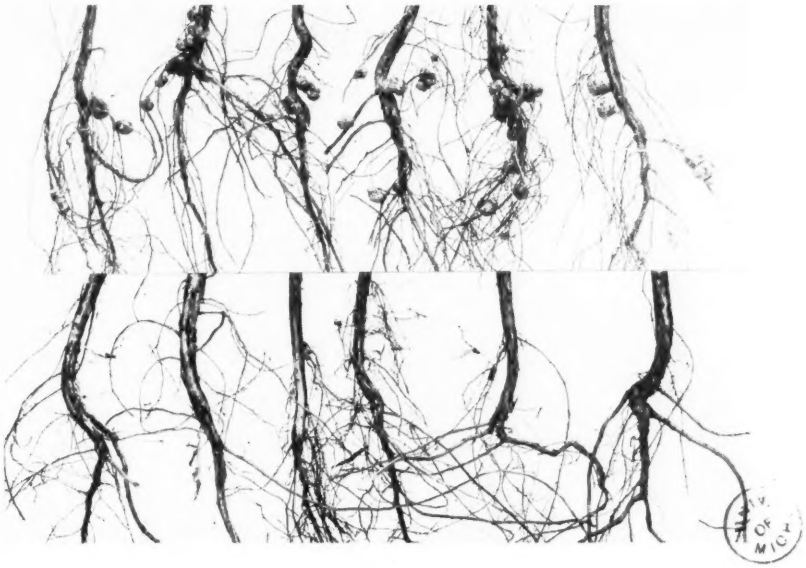

FIG. 2

PLATE 3

SOYBEAN GROWTH ON PUTNAM SILT LOAM AS INFLUENCED BY CALCIUM TREATMENTS

From left, culture, no treatment, culture and acid phosphate, culture and 3 tons limestone

DECOMPOSITION OF CITRIC ACID BY SOIL

L. A. DEAN AND A. L. DEAN

Experiment Station, Association of Hawaiian Pineapple Canners; University of Hawaii

Received for publication April 22, 1929

It is well recognized that most soils "fix" the phosphate ions added in the form of water-soluble phosphates, and that the soil solution is poor in phosphorus. On the other hand, many soils have the property of yielding water extracts with about the same content of phosphates to many successive extractions. Some simple laboratory method of measuring quickly the amount of phosphates which the soil will readily supply would be of great utility. For many years the method proposed by Dyer (1) has been widely used for the determination of "available" phosphates in soils. This method consists in extracting the soil with a 1 per cent solution of citric acid under conditions allowing a thorough interaction of solvent and soil with subsequent analysis of the extract. Dyer's original idea was that the acidity of this solvent closely resembled that of the cell sap of roots. His method has persisted, albeit the acidity of the cell contents of roots now appears irrelevant.

In the earlier procedure 200 gm. of air-dry soil is placed in a bottle with 2 liters of water containing 20 gm. of citric acid, and the mixture allowed to remain in contact for a week with many daily shakings, so that some 400 shakings in all are given. The solution is subsequently filtered off and analyzed for various ions, notable potassium and phosphate. We have employed the modified method in which 150 gm. of soil and 1500 cc. of 1 per cent citric acid solution are placed in 2-liter bottle, the stopper secured, and the bottle tumbled for 6 hours in a mechanical shaking device.

It has been somewhat generally accepted that any soil yielding less than 0.01 per cent of P_2O_5 by this citric acid method is in need of phosphatic fertilizers (3). The results of the analyses of 272 Hawaiian soils used for pineapple growing are shown in the graph. It is quite evident that soils analyzing as high as 100 p.p.m., (0.01 per cent) are distinctly uncommon. The mean is 29.4 p.p.m., less than one-third of Dyer's minimum. Our soils, however, give no such general indications of phosphate deficiency as this comparison would indicate.

In the course of a study of the behavior of phosphatic fertilizers in the soil of several representative pineapple fields we have found reasons seriously to question the applicability of the citric acid method.

The question being studied involved the difference in available phosphate which would be found in a given soil following the addition of equivalent amounts of phosphate in the form of finely ground raw rock on the one hand

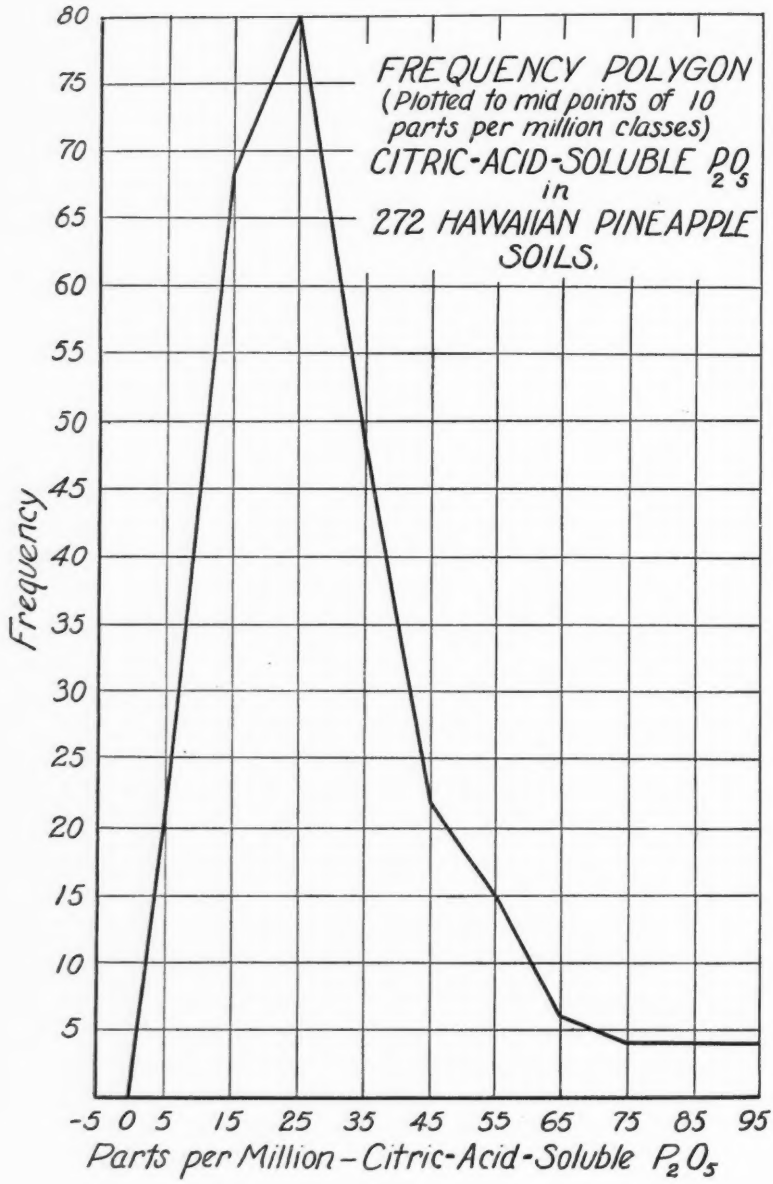
FREQUENCY POLYGON
(Plotted to mid points of 10 parts per million classes)
CITRIC-ACID-SOLUBLE P_2O_5
in
272 HAWAIIAN PINEAPPLE SOILS.
Frequency
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
-5 0 5 15 25 35 45 55 65 75 85 95
Parts per Million – Citric-Acid-Soluble P_2O_5

FIG. 1

and of superphosphate on the other. Thirty pounds of soil from pineapple fields in three representative districts on the island of Oahu were carefully mixed with the calculated amounts of the phosphates and placed in tubs. The amounts of the fertilizers corresponded to 1821 pounds of raw rock and 2810 pounds of superphosphate per acre, equivalent to 200 p.p.m. of water-soluble P_2O_5 from superphosphate and the same amount of total P_2O_5 from raw rock, calculated on a basis of 3,000,000 pounds of soil per acre. The moisture content of the soil was raised to about 30 per cent (the moisture equivalent of these soils is around 35 per cent) and the phosphates and soils were allowed to react.

SOIL	PHOSPHATE ADDED	CITRIC-SOLUBLE P_2O_5	
		After 7 days	After 14 days
		p.p.m.	*p.p.m.*
H. P. 48	None	80	68
	Raw rock	149	138
	Superphosphate	143	143
C. P. C. 123	None	55	57
	Raw rock	124	108
	Superphosphate	128	103
A. H. P. C. 1	None	21	20
	Raw rock	23	26
	Superphosphate	33	36

The results obtained indicate a sharp difference in behavior between two of the soils and the third one. Whereas the addition of phosphates caused a marked increase in the citric-soluble phosphate of the first two, the last was but slightly affected. This last soil is one of the high manganese soils characteristic of certain areas devoted to pineapple growing. A test was made of two other soils containing manganese equivalent to more than 3 per cent Mn_3O_4. One of these showed a rise of 75 p.p.m. of citric-acid-soluble P_2O_5 following the addition of 200 p.p.m. in the form of superphosphate, the other showed a rise of 22. This indicated no consistent behavior on the part of the high manganese soils.

However little virtue one can see in the use of 1 per cent citric acid because it approximates the acidity of the sap of roots, it does have certain features to recommend it. Citric acid is a slightly dissociated acid and as a consequence a substantial portion of the acid used in the extraction could be neutralized, and the pH of the resulting solution not materially affected. It would, therefore, be rational to assume that by using 1 per cent citric acid one was employing an extracting medium calculated to maintain a mild acidity within narrow limits. Teakle (4) has pointed out the marked differences in solubility of the relatively insoluble soil phosphates due to variations in the pH of the solvent water. To get comparable results with various soils, it is,

therefore, highly desirable that they be extracted with a solvent which will maintain a narrow range of pH in contact with all soils, unless, indeed, one wishes to allow for the effect of the natural pH of the soil and therefore uses distilled water. Our confidence in the uniformity of the pH of citric acid extractions was considerably shaken when determinations were run on two samples and they were found to be far from the 2.2 value of a 1 per cent citric acid solution.

As a result samples from representative pineapple fields on the island of Oahu and one from Molokai were treated with 1 per cent citric acid in accordance with the regular procedure and at the end of the period of shaking the pH of each was determined. The results follow:

Field		*pH of citrated acid extract*
H. P. Co.	3 C	2.9
	48	3.7
	49	4.6
	24 A	2.3
	75	6.0
	76	4.1
	59 A	3.6
C. P. C.	139	3.9
	123	4.0
Libby	40 C	2.9
	107	3.0
	Molokai 1	3.1
A. H. P. C.	Station 1	6.0

These results show an extraordinary variation. Instead of extracting all soils at uniform pH we have been doing nothing of the sort. The results of 1 per cent citric acid analyses of different soils are not comparable.

We undertook to find out how strong citric acid solution would be required to get a low pH with the Station Field 1 soil, and how much difference the increased acid would make in the dissolved phosphate.

Solvent	*pH*	*P_2O_5 extracted per cent*
1 per cent citric acid	6.0	0.0012
2 per cent citric acid	5.5	0.0037
4 per cent citric acid	3.8	0.0169
8 per cent citric acid	2.9	0.0339

It was noted that a very high gas pressure develops in the bottles in which some soils are shaken with citric acid. This was especially noticeable in the series just reported—in fact a mud shower bath attended the opening of one of them. A test of the gas showed it to be carbon dioxide. That this was not due to the decomposition of carbonates was shown by the fact that when this same soil was shaken with 1 per cent hydrochloric acid no gas was formed and the pH of the acid remained unchanged. The apparent conclusion is that citric acid is decomposed by the soil with the formation of carbon dioxide as one of the products.

The Hawaiian soils, aside from some few which are of coral origin, have developed through the degradation of basaltic lavas and ashes high in iron. The so-called lateritic soils of tropical regions are reported to be high in ferric oxides. The color of many of the Hawaiian soils is decidedly red. The paper of Fry and Gerwe (2) dealing with the decompositon of citric acid in the presence of light and iron salts was suggestive. They found that in the presence of ferric salts and light, citric acid decomposes to form acetone and carbon dioxide.

To avoid the possibility of the evolution of CO_2 due to biological activities in the soil, two samples of soil of 40 gm. each contained in Erlenmeyer flasks were sterilized in the autoclave and mixed with 400 cc. of water containing in one flask 4 gm. of citric acid and in the other an equivalent amount of hydrochloric acid. The flasks were provided with two-hole stoppers and a gentle stream of air which had passed through KOH was aspirated through the contents of the flasks and then, after being dried, through a CO_2 absorbing bulb. The weight of CO_2 evolved with the citric acid in four hours was .4767 gm., with hydrochloric acid it was .0016 gm.

The citric acid solution which had been shaken with soil from Field 1 of the Station in the regular analytical procedure was distilled and the distillate tested for acetone by Gunning's test. A yellow precipitate and the characteristic odor of iodoform were readily obtained. A similar distillate was prepared and tested for acetone by the preparation of dibenzylacetone (melting point, 110°–112°C.). The product obtained melted at 109.5°, and a similar product obtained from pure acetone melted at 108°. The mixture of the two melted at 108.5°. When the experiment was repeated using for the shaking a bottle painted black, gas was evolved and a sample of dibenzylacetone melting at 108.5° obtained from the distillate.

From the results just detailed we are convinced that some of our Hawaiian soils have the property of decomposing citric acid solutions to such a degree as seriously to affect the value of this solvent. Two of the products of decomposition are carbon dioxide and acetone, which, in the light of the work of Fry and Gerwe, are presumptive evidence of the activity of the iron compounds of the soil.

As a partial check on the phosphate-supplying power of the three soils reported on in the preceding, five tubs were filled with each soil, phosphates well mixed in according to the schedule, and cowpeas planted. Fifteen plants were allowed to grow in each tub for 90 days. The appearance of the plants at the end of the growing period is shown in plate 1, and the analytical results are listed in the table on following page.

There is no consistent relation between the amount of phosphorus the cowpeas could remove and that extracted by 1 per cent citric acid. On the other hand, the soil yielding the lowest citric acid extract furnished the most phosphate to the plants, and at the same time had such a destructive action on citric acid that the pH of its 1 per cent solution was raised to 6.0 during the analytical extraction. The low citric-soluble phosphate of our group of Hawaiian pineapple soils is very possibly related to the loss of acidity of the citric acid during

the procedure. However valuable the Dyer method may be as a measure of "available" phosphate in soils in other countries, we are convinced that it is wholly unreliable under our conditions.

FIELD	TOTAL P_2O_5 IN ORIGINAL SOIL	FERTILIZER		CITRIC-SOLUBLE P_2O_5	DRY WEIGHT COWPEAS PER TUB	P_2O_5 PER TUB
		Kind	P_2O_5			
	per cent		*p.p.m.*	*p.p.m.*	*gm.*	*gm.*
H. P. Co. 48	0.284	None	None	70	41	0.111
		Raw rock	200	140	43	0.109
		Raw rock	400	—*	47	0.125
		Super	200	143	62	0.197
		Super	400	—*	72	0.326
C. P. C. 123	0.265	None	None	56	17	0.057
		Raw rock	200	116	48	0.151
		Raw rock	400	—*	52	0.179
		Super	200	115	53	0.213
		Super	400	—*	55	0.250
Station 1	0.362	None	None	20	50	0.211
		Raw rock	200	24	53	0.228
		Raw rock	400	—*	41†	0.244
		Super	200	34	55	0.326
		Super	400	—*	86	0.386

* Not determined.

† 14 plants only.

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PLATE 1

COWPEAS

From top to bottom: H. P. Co. Field No. 48
C. P. C. Field No. 123
A. H. P. C. Field No. 1

Right to left: 1. No treatment
2. Raw rock 200 p.p.m. P_2O_5
3. Raw rock 400 p.p.m. P_2O_5
4. Superphosphate 200 p.p.m. P_2O_5
5. Superphosphate 400 p.p.m. P_2O_5

THE RELATION BETWEEN THE ABSORBED AND THE EXCHANGEABLE CALCIUM AND MAGNESIUM CONTENT OF A SOIL FOUR YEARS AFTER ADDITIONS

W. H. MacINTIRE AND K. B. SANDERS

The University of Tennessee Agricultural Experiment Station

Received for publication April 25, 1929

The subject of exchangeable bases in the soil has been dealt with in numerous articles during recent years. Special stress has been placed upon the replaceability of calcium. A number of methods and modifications have been advanced to differentiate between those bases and alkaline-earths that have passed into combination with the clay-humus complexes and those that are still present in mineral, or non-exchangeable, form.

The contributions hitherto made have been mainly studies upon the bases that were native to the soil. It is of interest and value to study the availability of the calcium that has withstood the agencies of weathering; but, if liming is necessary, the fate of added liming materials is even more important, since the availability, or replaceability, of the absorbed calcium and magnesium that is derived from economic additions is probably a primary factor in any sustained benefit from liming.

EXPERIMENTAL

The plan of the present study was to determine the replaceable, or exchangeable, calcium and magnesium content of a soil that had absorbed or "fixed" known quantities (12) from certain additions that had been made four years previously in outdoor lysimeter studies. The method of Hissink (5) was decided upon as being a recognized procedure for the determination of exchangeable bases.

The soil used was a brown loam that had been under control and subject to outdoor conditions in $\frac{1}{20,000}$-acre lysimeters from 1921 to 1925. Thirteen different calcic and magnesic treatments of constant CaO equivalence had been made in the upper and also in the lower zones, or halves. The two zones were demarcated by a wire-cloth disc. In the series where the treatments had been incorporated only with the upper half of the 8-inch depth of soil, the percolates had passed through an underlying untreated layer. In a parallel series the upper zone had received no additions and the percolates from the lower treated zone had passed directly into the percolate receptacles. The fraction of each addition that was still present as a non-hydroxide and non-carbonate compound was designated as that which had been absorbed

or "fixed" by the soil. This fraction was determined by subtracting from the $CaCO_3$-equivalence of each treatment the sum of its carbonate residue and calcium + magnesium outgo.

At the end of the four years of exposure each zone of each tank was removed, thoroughly mixed, and air-dried, and a 2-quart sample preserved. Charges of 25 gm. of a 2-mm. sieving were used to determine the calcium and magnesium extracted by the leachates of normal sodium chloride. Parallel preliminary extractions were also made with solutions of normal ammonium chloride. The ammonium chloride solution exerted greater solvent action upon those particles of high-calcic and dolomitic limestone that still remained where the coarser separates had been added. This is in harmony with Hissink's observations (5) relative to the solvent action exerted by the two chlorides upon calcium carbonate. The different caustic, limestone, and dolomite treatments are given in each table, along with the calcium-magnesium data that are reported on a basis of $CaCO_3$ equivalence.

THE RECOVERIES OBTAINED BY THE HISSINK PROCEDURE

Unlimed controls

Comparisons between the Hissink-method data are made against the average of the controls that had been exposed for four years and also against the original soil that had been preserved, under seal, in the laboratory. The data of table 1 show a decrease of 939 pounds of exchangeable calcium + magnesium for the full depth of unsupplemented soil for the 4-year period. This decrease was accounted for by a loss of 353 pounds from the upper half, and 586 pounds from the lower half. The total calcium-magnesium that had been found in the percolates from the control tanks during this period was 1234 pounds—898 pounds attributable to calcium and 336 to magnesium (7). This represents a ratio of 2.67:1 in the outgo, whereas extractions from the reserve sample of the original soil gave, by the Hissink method, an exchangeable calcium-magnesium ratio of 5.68 to 1. The corresponding ratios for the upper and lower zones of the exposed soil after four years, without additive treatment, were 5.32:1 and 7.75:1, respectively. Hence, the exchangeable calcium-magnesium was apparently 395 pounds less than the actual outgo of the two alkaline-earth bases; but the analysis of rain-gauge waters showed that the rainfall had supplied 296 pounds of soluble calcium-magnesium salts. It is therefore evident that there is a marked difference between the ratio of calcium to magnesium in the natural percolates, and that found in the sodium chloride leachates from the unlimed soil. The proportions of magnesia in the natural leachings are much greater than those found in the sodium chloride extractions.

The decrease in the exchangeable calcium-magnesium content of the lower zone of the exposed controls was considerably greater than that shown by the upper zone, in a comparison with the original soil. This evidence of

TABLE 1

The exchangeable calcium + magnesium present in a naturally leached brown loam four years after additions of various liming materials to the surface zone, as determined by the Hissink Method—terms of $CaCO_3$ equivalence*

TANK NUMBER	TREATMENTS EQUIVALENT TO 3570 POUNDS $CaCO_3$ PER ACRE	SOIL THAT RECEIVED THE ADDITIONS						SOIL THAT UNDERLAY THE TREATED SOIL						FULL DEPTH BOTH ZONES		
		Analysis air-dry soil			Pounds Ca + Mg upper zone or half, 1,000,000 pounds moisture-free soil			Analysis air-dry soil			Pounds Ca + Mg upper zone or half, 1,000,000 pounds moisture-free soil			Pounds Ca + Mg per 2,000,000 pounds moisture-free soil		
		Ca	Mg	Ca + Mg	Found	Increase over		Ca	Mg	Ca + Mg	Found	Increase over		Found	Increase over	
						Original soil	Exposed controls					Original soil	Exposed controls		Original soil	Exposed controls
		per cent	*per cent*	*per cent*				*per cent*	*per cent*	*per cent*						
80 A + B	Controls	0.218	0.041	0.259	2,617	−353	.....	0.209	0.027	0.236	2,384	−586	...	5,001	−939	.....
84	Hydrated Lime	0.394	0.046	0.440	4,445	1,475	1,828	0.291	0.023	0.314	3,172	202	788	7,617	1,677	2,616
85	Burnt Dolomite	0.295	0.127	0.422	4,263	1,293	1,646	0.240	0.077	0.317	3,203	233	819	7,466	1,526	2,465
86	Dolomite-oxide mixture	0.303	0.125	0.428	4,324	1,354	1,707	0.237	0.081	0.318	3,213	243	829	7,537	1,597	2,536
88	Limestone, 10–20 mesh	0.367	0.045	0.412	4,162	1,192	1,545	0.228	0.040	0.268	2,708	−262	324	6,870	930	1,869
89	Limestone, 20–40 mesh	0.393	0.044	0.437	4,415	1,445	1,798	0.273	0.029	0.302	3,051	81	667	7,466	1,525	2,465
90	Limestone, 40–80 mesh	0.403	0.035	0.438	4,425	1,455	1,808	0.286	0.025	0.311	3,142	172	758	7,567	1,627	2,566
91	Limestone, 80–200 mesh	0.385	0.040	0.425	4,294	1,324	1,677	0.307	0.018	0.325	3,283	313	899	7,577	1,637	2,576
87	Limestone, Composite†	0.367	0.050	0.417	4,213	1,243	1,596	0.264	0.024	0.288	2,910	−60	526	7,123	1,183	2,122
93	Dolomite, 10–20 mesh	0.273	0.100	0.373	3,768	798	1,151	0.215	0.036	0.251	2,536	−434	152	6,304	364	1,303
94	Dolomite, 20–40 mesh	0.300	0.106	0.406	4,102	1,132	1,485	0.232	0.051	0.283	2,859	−111	475	6,961	1,021	1,960
95	Dolomite, 40–80 mesh	0.319	0.114	0.433	4,375	1,405	1,758	0.242	0.061	0.303	3,061	91	677	7,436	1,496	2,435
96	Dolomite, 80–200 mesh	0.322	0.111	0.433	4,375	1,405	1,758	0.250	0.061	0.311	3,142	172	758	7,517	1,577	2,516
92	Dolomite, Composite†	0.292	0.109	0.401	4,051	1,081	1,434	0.230	0.052	0.282	2,849	−121	465	6,900	960	1,899
	Unexposed soil, preserved air-dry	0.250	0.044	0.294	2,970	.....	.....	.....	.....	.....	.....	.....	.....	5,940	.....	.....

* Using Normal NaCl.

† Composed of equal parts of 10–20-, 20–40-, 40–80-, and 80–200-mesh separates.

greater loss from the lower zone is in harmony with findings relative to a more intensive evolution of CO_2 in the lower zone (15), which is usually more moist.

The replacement of magnesium by sodium was practically constant for these upper-zone units to which no magnesium was added. The replacements from the lower-zone units, however, were less than those from the controls, save for the coarsest limestone separate of 10–20-mesh. This may be due to the previous replacement and leaching of magnesium by the neutral calcium salts that had been engendered in the upper zone of treatment during the four years of exposure (7, 8).

Surface-zone additions

The data of table 1 show the enhancements that were registered by the Hissink method for the limed upper zone and also those found in the underlying one. Every surface-zone addition caused an increased supply of exchangeable calcium-magnesium, on the basis of full depth of soil, in comparison with both the original soil and the exposed controls. This was not true, however, in a comparison between the underlying untreated zone and the original soil. Each underlying layer showed an increased calcium-magnesium content over that of the lower zone of the exposed controls, but the increments brought by percolates from the less rapidly disintegrated, coarsest limestone separate and composite were insufficient to offset the difference between the sum of the exchangeable calcium and magnesium originally present and that found in the underlying untreated zone after four years. This held also for the 10–20-mesh, 20–40-mesh, and composite dolomite additions. The average ratio between the enhanced amounts of calcium-magnesium for the upper zone that was in direct contact with the surface-zone additions and those enhancements found for the lower zone was 2.6 to 1. In the eight instances where increments to the underlying zone were larger than the respective amounts lost from the untreated soil during the 4-year period there was an average increase of 188 pounds, whereas the corresponding enhancement average for the upper zone was 1395 pounds. This gives an upper-zone to lower zone ratio of 7.4 to 1. For the full depth of soil the 13 additions showed an average gain of 1317 pounds in exchangeable alkaline-earth bases, in comparison with the original soil, and 2256 pounds against the exposed soil. The enhancements derived from the equivalent additions of high-calcic and dolomitic limes, 20–40-, 40–80-, and 80–200-mesh limestones, and the 40–80-mesh dolomite separates are in close agreement. The tendency of the soil to absorb somewhat more limestone than dolomite, or rather to absorb the former more rapidly, is shown by comparisons between the two limestone groups in the last column. This differential is at a minimum, however, in the case of the 80-mesh products.

Subsurface-zone additions

In table 2 are given the recoveries of exchangeable calcium and magnesium from the additions to the lower zone. Previous studies and observations with these tanks warrant the assumption that the several units of the upper untreated zone may be considered as a constant. In this series there was no catch zone and the calcium-magnesium losses through leaching had been consistently greater (7, 8) than those from the series where additions were made

TABLE 2

The exchangeable calcium + magnesium present in a naturally leached brown loam four years after additions of various liming materials to the subsurface zone, as determined by the Hissink Method—terms of $CaCO_3$ equivalence

TANK NUMBER	TREATMENTS EQUIVALENT TO 3570 POUNDS $CaCO_3$ PER ACRE	ANALYSIS AIR-DRY SOIL			Ca + Mg IN ZONE, MOISTURE-FREE BASIS		
		Ca	Mg	Ca+Mg	Found	Increase over	
						Original soil	Leached controls
		per cent	*per cent*	*per cent*	*pounds*	*pounds*	*pounds*
80 A + B	Controls	0.209	0.027	0.236	2,384	−586	.....
84	Hydrated Lime	0.416	0.023	0.439	4,435	1,465	2,051
85	Burnt Dolomite	0.341	0.092	0.433	4,375	1,405	1,991
86	Dolomite-oxide mixture	0.352	0.091	0.443	4,476	1,506	2,092
88	Limestone, 10–20 mesh	0.406	0.028	0.434	4,385	1,415	2,001
89	Limestone, 20–40 mesh	0.425	0.025	0.450	4,546	1,576	2,162
90	Limestone, 40–80 mesh	0.419	0.029	0.448	4,526	1,556	2,142
91	Limestone, 80–200 mesh	0.409	0.028	0.437	4,415	1,445	2,031
87	Limestone, Composite*	0.408	0.030	0.438	4,425	1,455	2,041
93	Dolomite, 10–20 mesh	0.304	0.081	0.385	3,890	920	1,506
94	Dolomite, 20–40 mesh	0.335	0.083	0.418	4,223	1,253	1,839
95	Dolomite, 40–80 mesh	0.349	0.090	0.439	4,435	1,465	2,051
96	Dolomite, 80–200 mesh	0.345	0.095	0.440	4,445	1,475	2,061
92	Dolomite, Composite*	0.339	0.091	0.430	4,344	1,374	1,960
	Unexposed Soil	0.250	0.044	0.294	2,970	.....	.....

* Composed of equal parts of 10–20-, 20–40-, 40–80-, and 80–200-mesh separates.

to the upper zone. It follows that there was a smaller quantity of residual exchangeable bases to be extracted by the sodium chloride treatment. On the other hand, the disintegration of the limestone and dolomite separates was much more extensive in the lower zone (11); hence, rather uniform values obtained for 12 of the 13 caustic and limestone and dolomite additions, the one exception being the coarsest, or 10–20-mesh, dolomite separate.

The enhancements found by the Hissink method show, for the 13 lower-zone additions, an average of 1385 pounds over the original soil and a corre-

sponding average of 1994 pounds in excess of the final exchangeable calcium-magnesium content of the exposed soil. This average of 1994 pounds was from a series that gave, through natural leaching, a loss of 850 pounds in excess of that from the controls, whereas, in the case of the 13 surface-zone additions the average enhancement of 2266 pounds represented the full-depth effect where the calcium-magnesium loss had been only 155 pounds in excess of that from the controls.

TABLE 3

The exchangeable Ca + Mg, determined by the Hissink Method four years after surface-zone additions of various liming materials, as compared with the "fixed" Ca + Mg determined in lysimeter studies by a balance between additions, outgo, and carbonate residues—terms of $CaCO_3$ equivalence

TANK NUMBER	TREATMENT*	EFFECT EXERTED BY BOTH UPPER AND LOWER ZONES						
		Carbonate residues from additions in soil analyzed	Four-year outgo Ca + Mg over that of controls	Increase in non-carbonate Ca + Mg				Ratio between determined occurrences of the "fixed" and the exchangeable Ca + Mg
				Determined as "fixed"† in lysimeter studies		Determined as exchangeable by the Hissink Method		
		pounds	*pounds*	*pounds*	*per cent of addition*	*pounds*	*per cent of addition*	
84	Hydrated Lime	160	271	3,139	87.9	2,616	73.3	1.20 to 1
85	Burnt Dolomite	140	231	3,199	89.6	2,465	69.0	1.30 to 1
86	Dolomite-oxide mixture	140	156	3,274	91.7	2,536	71.0	1.29 to 1
88	Limestone, 10–20 mesh	1,670	78	1,822	51.0	1,869	52.4	0.97 to 1
89	Limestone, 20–40 mesh	270	170	3,130	87.7	2,465	69.0	1.27 to 1
90	Limestone, 40–80 mesh	40	211	3,319	93.0	2,566	71.9	1.29 to 1
91	Limestone, 80–200 mesh	100	226	3,244	90.9	2,576	72.2	1.26 to 1
87	Limestone, Composite‡	650	51	2,869	80.4	2,122	59.4	1.35 to 1
93	Dolomite, 10–20 mesh	1,970	38	1,562	43.8	1,303	36.5	1.20 to 1
94	Dolomite, 20–40 mesh	870	105	2,595	72.7	1,960	54.9	1.32 to 1
95	Dolomite, 40–80 mesh	510	152	2,908	81.5	2,435	68.2	1.19 to 1
96	Dolomite, 80–200 mesh	150	207	3,213	90.0	2,516	70.5	1.28 to 1
92	Dolomite, Composite‡	1,040	115	2,415	67.6	1,899	53.2	1.27 to 1

* Each treatment = to 3570 pounds of $CaCO_3$.

† The $CaCO_3$-equivalent addition minus the sum of the carbonate increase and enhancement in outgo.

‡ Composed of equal parts of 10–20-, 20–40-, 40–80-, and 80–200-mesh separates.

COMPARISON BETWEEN HISSINK-METHOD RESULTS AND LYSIMETER ABSORPTION DATA

Surface-zone additions

The data of table 3 give comparisons between the amounts of calcium-magnesium that had been absorbed or "fixed" by the two zones of full soil

depth from the 13 upper-zone additions and the corresponding increases in the exchangeable calcium-magnesium registered by the Hissink procedure. From the constant 3570-pound equivalence of $CaCO_3$ (2000 pounds CaO) was deducted the respective sums of carbonate increases and enhanced outgo, in order to secure the data of the third column. The "fixed," or absorbed, data are higher than the Hissink-method values, without exception. The

TABLE 4

The exchangeable Ca + Mg, determined by the Hissink Method four years after subsurface additions of various liming materials, as compared with the "fixed" Ca + Mg determined in lysimeter studies by a balance between additions, outgo, and carbonate residues—terms of $CaCO_3$ equivalence

TANE NUMBER	TREATMENT*	EFFECT EXERTED BY A CONSTANT UNTREATED SURFACE ZONE AND THE LOWER ZONE OF ADDITIONS						
		Carbonate residues from additions in soil analyzed	Four-year outgo Ca + Mg over that of controls	Increase in non-carbonate Ca + Mg				Ratio between determined occurrences of the "fixed" and the exchangeable Ca + Mg
				Determined as "fixed"† in lysimeter studies		Determined as exchangeable by the Hissink Method		
		pounds	*pounds*	*pounds*	*per cent of addition*	*pounds*	*per cent of addition*	
84	Hydrated Lime	140	1,148	2,282	63.9	2,051	57.5	1.11 to 1
85	Burnt Dolomite	200	1,215	2,155	60.4	1,991	55.8	1.08 to 1
86	Dolomite-oxide mixture	200	1,090	2,280	63.9	2,092	58.6	1.09 to 1
88	Limestone, 10–20 mesh	860	506	2,204	61.7	2,001	56.1	1.10 to 1
89	Limestone, 20–40 mesh	170	817	2,583	72.4	2,162	60.6	1.19 to 1
90	Limestone, 40–80 mesh	000	1,098	2,472	69.2	2,142	60.0	1.15 to 1
91	Limestone, 80–200 mesh	000	1,133	2,437	68.3	2,031	56.9	1.20 to 1
87	Limestone, Composite‡	340	900	2,330	65.3	2,041	57.2	1.14 to 1
93	Dolomite, 10–20 mesh	1,550	153	1,867	52.3	1,506	42.2	1.24 to 1
94	Dolomite, 20–40 mesh	620	486	2,464	69.0	1,839	51.5	1.34 to 1
95	Dolomite, 40–80 mesh	160	805	2,605	73.0	2,051	57.5	1.27 to 1
96	Dolomite, 80–200 mesh	110	1,013	2,447	68.5	2,061	57.7	1.19 to 1
92	Dolomite, Composite‡	700	689	2,181	61.1	1,960	54.9	1.11 to 1

* Each treatment = 3570 pounds $CaCO_3$.

† The $CaCO_3$-equivalent addition minus the sum of the carbonate increase and enhancement in outgo.

‡ Composed of equal parts of 10–20-, 20–40-, 40–80-, and 80–200-mesh separates.

average ratio between the calcium-magnesium absorption data and the Hissink-method recoveries is 1.24:1, with rather close agreement for 12 of the 13 units. The lysimeter results give an average "fixation," or absorption, of 2822 pounds. This is equivalent to 79.1 per cent of the constant value of the additions, whereas the average enhancement in exchangeable calcium-magnesium by the Hissink procedure was 2178 pounds, or 63.2 per cent.

Subsurface-zone additions

The data of table 4 give corresponding results from the series that received the additions to the lower zone. The lysimeter data show that an average of 2324 pounds, or 65.3 per cent of the 3570-pound $CaCO_3$-equivalent additions, was still present in the non-carbonate form, whereas the average of the enhancements registered by the Hissink procedure was 1994 pounds, or 55.9 per cent. The average ratio between the "fixed," or absorbed, bases and the enhancement obtained by the Hissink method is 1.09 to 1. In this zone of greater carbonate decomposition, greater outgo through percolations, and lesser retention, the results by the two methods of attack are more nearly in accord.

GENERAL DISCUSSION

A closer concordance would be expected if both methods truly record the "fixed," or absorbed, fractions of the additions after a period of four years of exposure to weather. It was thought possible that the mechanics of the Hissink procedure might be altered so as to effect a greater recovery and, hence, a closer agreement in the results obtained by the two methods. The extractions were therefore repeated after preliminary dispersion had been effected by means of the electric device proposed by Bouyoucos (3), and modified by Baver (2). Special glass containers with rubber covers and vertical glass baffle rods were used to effect maximum dispersion. The dispersed systems were then filtered as in the regular Hissink procedure. Gravitational filtration was greatly retarded as a result of the preliminary dispersion, but the results for both calcium and magnesium were practically identical with those obtained by the standard procedure. A detailed presentation of these data would represent merely a duplication of those already given. One indication developed from this comparison. Variation in methods of manipulation may appreciably alter the amounts of manganese that will be extracted by the leachates. It is advisable, and may be necessary, to remove this element, along with iron and aluminum, by means of ammonium persulfate, prior to the precipitation of calcium.

It has been assumed that the process used in the Hissink procedure would recover the full amount of the exchangeable bases, calcium more especially. Moreover, the possibility that the NaCl solution might exert an appreciable solvent action upon "acid-soluble" mineral non-complexes has been considered, and by Hissink himself (5, p. 272). He logically contended that any such slight solvent action, and plus error, would not materially influence the value of results obtained by the use of his method. The plus error attributable to the solubility of alkaline-earth carbonates is admitted and provided for (5, p. 271).

The Hissink procedure gives concordant determinations and these evidently register some definite property. This method, and similar procedures, have their place and value, and the principle involved is not being condemned.

The method may truly determine the amount of *native* bases that are components of the exchange complex. It may also give a full recovery of those fractions of the lime additions that enter into true exchangeable or replaceable form. But the Hissink method uniformly failed to extract the full amounts of the non-carbonate residuals that had been derived from a 3570-pound $CaCO_3$ constant.

On the other hand, the lysimeter values may be considered as being close to the absolute. The neutralizing equivalence of each addition was determined by its titration value, supplemented by CaO and MgO determinations. The calcium-magnesium content of the percolates was determined by precision methods, whereas the carbonate-residue determinations were made by the Tennessee station (9), and official, method (1). This permits the use of 100-gm. charges in an especially adapted shaking device and insures extreme accuracy in the determination of minute amounts of carbonate-CO_2.

The relationship between the two series of data was somewhat uniform. This may mean one of two things, at least for this particular soil. Either (*a*) the Hissink method did not extract all of the absorbed or exchangeable calcium and magnesium that had been derived from additions, or (*b*) a part of the added calcium and magnesium entered into non-exchangeable or exceedingly resistant forms. When considered in the light of related data the second explanation appears more tenable.

Previous studies at the Tennessee station have shown that calcium and magnesium carbonates may react with different non-complex silicic and titanic materials (9, 6). It was especially emphasized that the reaction tendency of silicic acid, even in crystalline form, had received little attention and that it should not be disregarded. It was shown later that the addition of C. P. precipitated silica along with 4-ton additions of MgO resulted in a MgO-SiO_2 reaction and in the eradication of a lethal toxicity (15).

The products that result from reactions between calcium or magnesium and silica may not enter extensively into base-exchange reactions. If the pulverized, simple, natural silicate wollastonite may be used as an index of base-exchange reactions to be expected from combinations that ensue from contact between calcium compounds and colloidal silica, some of the calcium that is fixed by the soil would be expected to resist the solvent action of sodium chloride leachings. This was evidenced when a 5-gm. charge of wollastonite was mixed with 95 gm. of quartz and subjected to the Hissink procedure. In terms of $CaCO_3$-equivalence, the calcium content per liter was 22.4 mgm. whereas that of magnesium was 4.1 mgm. Serpentine gave corresponding extractions of 12.4 and 30.9 mgm.

The possibility of such a combination between added calcium compounds and soil components other than the exchange complex has also recently been suggested by Pierre (19) to account for certain observations made by him in a related type of work. In the case of repeated use of soluble potassic manures it has been shown that the added salts retreated into forms that were

not recoverable by base-exchange methods. The work of Frear and Erb at the Pennsylvania station (4), previous Tennessee station results (17), and the studies by Page and Williams (18) indicate very strongly that the continued use of soluble potassic salts results in the accumulation of combinations that are not dissolved out by procedures that are used to determine exchangeable bases.

Furthermore, the process of aging might be expected to decrease the solubility of absorption compounds, even if these were to remain constant in makeup, instead of passing into more complex forms. The factor of progressive decrease in solubility was suggested in previous data from this station (12), and further emphasized through calculations (9, p. 192) that indicated that absorbed calcium-magnesium had become progressively more resistant to percolation by rainwaters.

In the case of the present work, the time factor may be an important one. It seemed possible that the absorbed bases might have been recovered completely by such a procedure as the Hissink method, if the extractions had been made immediately, or shortly, after the absorption had taken place. This point was considered by the following minor experiment. Twenty-five-gram charges of soil were treated with a standard calcium hydroxide solution in an amount equivalent to a rate of 2000 pounds of CaO per 2,000,000 pounds of soil. This addition represented a small fraction of the absorptive capacity of the soil. The suspension was then evaporated to an air-dry condition, in a larger desiccator at room temperature and in a CO_2-free atmosphere. Three days were required for this treatment. The dry samples were then subjected to sodium chloride extractions by the Hissink procedure, in parallel with the untreated soil that had been used in the lysimeter studies. When the calcium that was removed by 2 liters of leachate from the original soil was subtracted from the amount obtained from the lime-treated charges, the recovery was 90 per cent of the addition. The unextracted 10 per cent fraction of the addition, however, amounted to only 0.01 per cent on the basis of soil charge. This tends to confirm the hypothesis that a reaction transpires between added lime and soil components other than the exchange complex.

One other possible factor may be of importance. It has been shown by Steenkamp (20) that the spontaneous dehydration of a soil exerts a marked influence upon its tendency to yield exchangeable bases by extraction methods, and that an increase or decrease is specific for a given soil. The lysimeter soils were spread and dried immediately after their removal from the tanks and kept in the air-dry condition under seal in the laboratory from 1925 to 1928. It is possible that, had the sodium chloride extractions been made upon the freshly taken samples, three years previously, the recoveries of the added calcium and magnesium might have been of different magnitude from those obtained after drying and aging. This possibility is not supported, however, by the results obtained when the Hissink method was used in an attempt to recover the full amount of added $Ca(OH)_2$ shortly after the addition had been made.

SUMMARY

The Hissink method was used to determine the enhancements in exchangeable calcium-magnesium from surface-zone and subsurface-zone incorporations of high-calcic and high-magnesic limes, limestone, and dolomite separates of a constant 3570-pound $CaCO_3$ equivalence that had been incorporated four years previously in 28 outdoor lysimeters. These findings were compared with the "fixation," or absorption, results obtained in the lysimeter studies.

The ratio of calcium to magnesium in the sodium chloride leachates from the original soil, and also that from the exposed controls, was decidedly greater than the one found in the rainwater percolates. The decrease in the exchangeable base content of the lower zone of the controls was materially greater than that found for the upper zone.

Each surface-zone addition caused an extraction of exchangeable bases in excess of that obtained from the surface zone of the original soil. This did not hold for the underlying unlimed zone in the case of the less extensively disintegrated limestone and dolomite separates.

The enhancements obtained by the Hissink method were uniform for the additions to the lower zone, where both natural-leaching losses and carbonate disintegration had been the greater.

The enhancements registered by the Hissink method were consistently less than the absorptions. The average "fixation" shown by the lysimeter studies for surface-zone incorporations was 79.1 per cent of the constant addition, whereas the enhancement registered by the Hissink procedure was 63.2 per cent. Corresponding values obtained by the two methods from the subsurface-zone incorporations were 65.3 and 55.9 per cent, respectively.

The average absorption from the 13 surface-zone incorporations was 1.25 times the calcium-magnesium enhancements found by the Hissink method. A corresponding figure of 1.17 was obtained for the subsurface-zone incorporations.

Preliminary dispersion failed to increase the recoveries obtained by the Hissink method.

The uniformly higher values for "fixation," or absorption, in the lysimeter studies indicated that a fraction of the additions had combined with soil components other than the exchange complex. This viewpoint was strengthened by citation of related work, previously reported, and by the incomplete recovery of freshly added $Ca(OH)_2$, in controlled atmosphere, by the technic of the Hissink method.

Addenda corrections are given for previous data relative to limestone and dolomite disintegration and "fixation."

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ADDENDA

A Correction in Calculations

for

"THE DISINTEGRATION OF LIMESTONE AND DOLOMITE SEPARATES, AS INFLUENCED BY ZONE OF INCORPORATION"[1]

and

"FIXATION OF CALCIUM-MAGNESIUM FROM BURNT LIMES, LIMESTONE AND DOLOMITE INCORPORATIONS IN TWO SOIL ZONES"[2]

By W. H. MacINTIRE and W. M. Shaw

In carrying out the studies reported in the foregoing paper a marked disparity appeared between the results obtained by the two methods of attack in certain instances. The analytical work was repeated and proved. It was then discovered that the carbonate-CO_2 results,[1] as given for 2,000,000 pounds had been multiplied by that amount instead of 1,000,000. The latter figure should have been used to obtain the occurrence for the 2,000,000-pound basis, since the additions had been incorporated in only one-half of the full weight of soil. The use of the data, thus erroneously computed in the first contribution,[1] to obtain the "fixation" data given in the second paper, introduced an error in those cases where residual carbonates remained from the coarser separates. In the case of complete absorption, no error was introduced and in the case of the smaller carbonate residues the error is not of appreciable magnitude, but the magnitude of the fixation data is affected in the case of the coarser limestone and dolomite separates. Since the error was a constant factor the general conclusions are not altered, but the magnitude of the amounts of coarser limestone and dolomite absorbed by the soil is considerably changed. The single table given in each paper is therefore reprinted with complete corrections.

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[1] *Soil Sci.* (1925) 20: 409.
[2] *Soil Sci.* (1926) 22: 111.

TABLE 1

Residual carbonates and disintegration of 2,000-pound CaO-equivalent limestone and dolomite separates from surface-zone and subsurface-zone incorporations in an acid Cumberland loam after exposure in lysimeter tanks for a period of 4 years*

SEPARATES INCORPORATED IN SOIL	$CaCO_3$—EQUIVALENT OF DETERMINED RESIDUAL CO_2					RESIDUAL $CaCO_3$ CORRECTED FOR CONTROLS			DISINTEGRATION OF ADDITION BY DIFFERENCE AS ACCOUNTED FOR BY ABSORPTION AND LEACHING	
	1	2	3	Average	Probable error	On basis of moisture-free soil: Analyses	On basis of moisture-free soil: Per 2,000,000 pounds	On basis of addition		
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*lbs.*	*per cent*	*per cent*	*lbs.*
Group 1. From limestone incorporated in surface zone										
10–20 mesh	0.191	0.192	0.194	0.192	±0.001	0.167	1,670	46.8	53.2	1,900
20–40 mesh	0.051	0.051	0.053	0.052	±0.001	0.027	270	7.6	92.4	3,300
40–80 mesh	0.028	0.026	0.033	0.029	±0.002	0.004	40	1.1	98.9	3,530
80–200 mesh	0.031	0.040	0.034	0.035	±0.003	0.010	100	2.8	97.2	3,470
Composite†	0.094	0.082	0.094	0.090	±0.004	0.065	650	18.2	81.8	2,920
Group 2. From limestone incorporated in subsurface zone										
10–20 mesh	0.100	0.109	0.113	0.107	±0.004	0.086	860	24.1	75.9	2,710
20–40 mesh	0.038	0.038	0.039	0.038	±0.001	0.017	170	4.8	95.2	3,400
40–80 mesh	0.022	0.023	0.019	0.021	±0.001	0.000	000	0.0	100.0	3,570
80–200 mesh	0.020	0.020	0.022	0.021	±0.001	0.000	000	0.0	100.0	3,570
Composite†	0.056	0.056	0.053	0.055	±0.001	0.034	340	9.5	90.5	3,230
Group 3. From dolomite incorporated in surface zone										
10–20 mesh	0.220	0.214	0.231	0.222	±0.005	0.197	1,970	55.2	44.8	1,600
20–40 mesh	0.113	0.108	0.116	0.112	±0.002	0.087	870	24.4	75.6	2,700
40–80 mesh	0.069	0.083	0.077	0.076	±0.004	0.051	510	14.3	85.7	3,060
80–200 mesh	0.033	0.041	0.033	0.036	±0.003	0.015	150	4.2	95.8	3,420
Composite†	0.123	0.131	0.133	0.129	±0.003	0.104	1,040	29.1	70.9	2,530
Group 4. From dolomite incorporated in subsurface zone										
10–20 mesh	0.162	0.179	0.188	0.076	±0.008	0.155	1,550	43.4	56.6	2,020
20–40 mesh	0.072	0.088	0.091	0.083	±0.006	0.062	620	17.4	82.6	2,950
40–80 mesh	0.032	0.036	0.044	0.037	±0.003	0.016	160	4.5	95.5	3,410
80–200 mesh	0.033	0.033	0.029	0.032	±0.001	0.011	110	3.1	96.9	3,460
Composite†	0.089	0.089	0.094	0.091	±0.002	0.070	700	19.5	80.5	2,870

* Constant of 2,000-pound CaO, or 3,570-pound $CaCO_3$ equivalences per acre.

† Equal quantities of 10–20-, 20–40-, 40–80-, and 80–200-mesh separates.

TABLE 1—*Concluded*

SEPARATES INCORPORATED IN SOIL	$CaCO_3$—EQUIVALENT OF DETERMINED RESIDUAL CO_2					RESIDUAL $CaCO_3$ CORRECTED FOR CONTROLS			DISINTEGRATION OF ADDITION BY DIFFERENCE AS ACCOUNTED FOR BY ABSORPTION AND LEACHING	
	1	2	3	Average	Probable error	On basis of moisture-free soil: Analyses	On basis of moisture-free soil: Per 2,000,000 pounds	On basis of addition		
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*lbs.*	*per cent*	*per cent*	*lbs.*
Group 5. From burnt lime controls in surface zone										
CaO	0.041	0.041	.....	0.041	.....	0.016	160	4.5	95.5	3,410
CaO–MgO‡	0.037	0.041	.....	0.039	.....	0.014	140	3.9	96.1	3,430
CaO–MgO§	0.037	0.041	.....	0.039	.....	0.014	140	3.9	96.1	3,430
Group 6. From burnt lime controls in subsurface zone										
CaO	0.038	0.031	.....	0.035	.....	0.014	140	3.9	96.1	3,430
CaO–MgO‡	0.041	0.041	.....	0.041	.....	0.020	200	5.6	94.4	3,310
CaO–MgO§	0.041	0.041	.....	0.041	.....	0.020	200	5.6	94.4	3,310
Group 7. From untreated controls										
Surface	0.026	0.026	0.024	0.025	±0.001	.....	.....	.....	.....	.....
Subsurface	0.020	0.019	0.025	0.021	±0.002	.....	.....	.....	.....	.....

‡ Burnt dolomite.

§ Mixture of separately calcined oxides.

TABLE 1

Fixation of Ca-Mg from a 3570-pound $CaCO_3$-equivalence (2000 pounds CaO) of $Ca(OH)_2$, CaO-MgO, and limestone and dolomite separates in surface-zone and subsurface-zone incorporations with a loam soil under outdoor conditions for a period of 4 years

Results are given in terms of $CaCO_3$-equivalence per 2,000,000 pounds of soil, moisture-free basis

TREATMENT	SURFACE-ZONE INCORPORATION					SUBSURFACE-ZONE INCORPORATION					FIXATION FROM SURFACE-ZONE INCORPORATION OVER THAT FROM SUBSURFACE INCORPORATION	
	Carbonate increase (1)	Total $CaCO_3$ equivalence accounted for by both fixation and leaching (2)	Total Ca-Mg outgo in excess of that from controls (3)	Fixation—Full depth of soil considered (4)	(5)	Carbonate increase (6)	Total $CaCO_3$ equivalence accounted for by both fixation and leaching (7)	Total Ca-Mg outgo in excess of that from controls (8)	Fixation in subsurface zone (9)	(10)	(11)	(12)
	pounds	*pounds*	*pounds*	*pounds*	*per cent*	*pounds*	*pounds*	*pounds*	*pounds*	*per cent*	*pounds*	*per cent*
$Ca(OH)_2$	160	3,410*	271	3,139	87.9¶	140	3,430	1,148	2,282	63.9¶	857	24.0¶
CaO-MgO†	140	3,430	231	3,199	89.6	200	3,370	1,215	2,155	60.4	1,044	29.2
CaO-MgO‡	140	3,430	156	3,274	91.7	200	3,370	1,090	2,280	63.9	994	27.8
L.S. 10–20	1,670	1,900	78	1,822	51.0	860	2,710	506	2,204	61.7	−382	−10.7
L.S. 20–40	270	3,300	170	3,130	87.7	170	3,400	817	2,583	72.3	547	15.4
L.S. 40–80	40	3,530	211	3,319	93.0	000	3,570	1,098	2,472	69.2	847	23.8
L.S. 80–200	100	3,470	226	3,244	90.9	000	3,570	1,133	2,437	68.3	807	22.6
L.S. Comp.§	650	2,920	51	2,869	80.4	340	3,230	900	2,330	65.3	539	15.1
Dol. 10–20	1,970	1,600	38	1,562	43.8	1,550	2,020	153	1,867	52.3	−305	−8.5
Dol. 20–40	870	2,700	105	2,595	72.7	620	2,950	486	2,464	69.0	131	3.9
Dol. 40–80	510	3,060	152	2,908	81.5	160	3,410	805	2,605	73.0	303	8.5
Dol. 80–200	150	3,420	207	3,213	90.0	110	3,460	1,013	2,447	68.5	766	21.5
Dol. Comp.§	1,040	2,530	115	2,415	67.6	700	2,870	689	2,181	61.1	234	6.5

* Assumption of theoretical carbonation.

† Calcined dolomite.

‡ Corresponding mixture of separately calcined CaO and MgO.

§ Equal parts of 10–20-, 20–40-, 40–80-, and 80–200-mesh separates.

¶ Basis of $CaCO_3$-equivalence of addition.

THE USE OF BACTERIOSTATIC DYES IN THE ISOLATION OF RHIZOBIUM LEGUMINOSARUM FRANK

IVAN A. ANDERSON[1]

Idaho Agricultural Experiment Station

Received for publication April 25, 1929

The place of dyes for the differentiating of various groups of bacteria has been studied quite extensively in recent years and various applications have been made of the new facts. An example of this is the use of eosin-methylene blue agar plates for the differentiation of *Escherichia coli* from its close relative, *Aerobacter aerogenes*. Another recent development in the use of the bacteriostatic effect of dyes upon certain groups of bacteria is for the purpose of ridding mixed cultures of undesired forms. This is done in the presence of nutrient media, so that the organism unaffected by the dye will outgrow the undesired forms. The tri-phenyl-methane group of dyes has been especially studied in this connection, and of these, crystal violet probably has received the most attention.

PREVIOUS STUDIES

Hall and Ellefson (5) found in the presumptive test for *Escherichia coli* that a concentration of $\frac{1}{100,000}$ of crystal violet in the culture medium would prevent the development of interfering anaerobes, while not inhibiting the growth of *Esch. coli*. In fact all gram-positive organisms were found to be less resistant to the action of the dye than were gram-negative organisms.

Skinner and Murray (7) found that the addition of crystal violet in a concentration of $\frac{1}{100,000}$ in the standard eosin methylene blue agar (American Public Health Association) inhibits the development of spreading colonies and in no way makes more difficult the identification or isolation of colon bacilli. On the contrary, the colonies of the colon bacilli were found to be even more typical than those on standard agar. Grossley (4) had previously shown that there was a direct correlation between gram-positiveness and the inhibition of growth by gentian violet, which is merely crystal violet with dextrin as an impurity. That is to say, gentian violet allowed gram-negative organisms to grow while it actually killed gram-positive organisms in the same concentrations.

Churchman (1) found that gram-negative organisms differ in their resistance to the bacteriostatic effect of crystal violet. As an example, he found that

[1] Submitted to the faculty of the graduate school, University of Idaho in partial fulfillment of the requirements for the degree of master of science. Published with consent of the director as Research Paper No. 62 from the Idaho Experiment Station.

¶ Basis of $CaCO_3$-equivalence of addition.

Eberthella typhi (Schröter) Buchanan is more resistant to crystal violet than *Esch. coli* (Escherich) Castellani and Chambers.

Vandecaveye (8), in a study of the gram-negative root nodule bacterium, *Rhizobium leguminosarum* (Frank), found that this organism was more resistant to the bacteriostatic action of the tri-phenyl-methane dyes than *B. radiobacter*, a common contaminant of cultures of *R. leguminosarum*.

PRESENT STUDIES

The common method of isolating *Rhizobium leguminosarum* is so tedious and uncertain in the hands of the inexperienced that a shorter and more efficient method is much to be desired. This is especially true in view of the difficulty of differentiating it from the common contaminant, *B. radiobacter*, which is very similar to *R. leguminosarum* in cultural and morphological characteristics. The differential resistance of *R. leguminosarum* and *B. radiobacter* to the action of the tri-phenyl-methane dyes offers a distinct possibility that these dyes and dyes belonging to other chemical groups may be of assistance in simplifying the method of isolation of the root nodule organism.

Dyes may be used for the isolation of an organism in several ways. One of these is to suspend the bacteria (mixed culture) in an aqueous solution of the dye for a period of time sufficient to kill the contaminating species and then transfer the survivors to a culture medium suitable for the growth of the desired species. Another method is to seed a mixed culture into a medium containing such a concentration of the dye that the growth of the undesired species will be inhibited while the desired species will grow sufficiently to produce visible colonies. The ideal dye for the isolation of legume root nodule bacteria would be one which could kill or retard in growth all of the organisms in the nodule except the desired one, *R. leguminosarum*. The following experiments were carried out with this in mind.

From the results of the investigations mentioned under "Previous Studies" it would seem that success would be more likely to result from the use of dyes of the tri-phenyl-methane group. However, only dyes which are water soluble could be used for the present studies. As classified by Conn (2) the following members of this group were used:

Basic Dyes: crystal violet, rosaniline hydrochloride, malachite green, basic fuchsin, methyl green, brilliant green, and methyl violet.
Acidic Dyes: acid fuchsin, acid green G, and methyl blue.

A few dyes belonging to other chemical groups also were used in a few experiments. These were as follows:

Quinone-Imide Group: methylene, blue and safranin. These are basic dyes.
Xanthene Group: eosin B, eosin Y, and rose bengal. These are all acidic dyes.
Azo Group: congo red.

All of the experiments reviewed here were carried on with crushed nodules or freshly isolated but badly contaminated cultures of alfalfa and bean strains of local origin. Old cultures were not used because preliminary studies had shown that organisms within the nodule are more resistant to dyes than are their descendants after the culture has been subjected for some time to growth under artificial conditions. A plausible explanation of this fact (not verified by microscopic studies) is based on the hypothesis that the organisms within the nodule are equipped with a heavier and thicker coating of slime than those of cultures grown artificially for some time. Fred (3) has shown that there is a correlation between the amount of slime production and the resistance of the organism to crystal violet. He also has shown that the alfalfa-sweet clover group is more susceptible to hydrogen-ion concentration than other physiological groups. This physiological group also is more susceptible to the bacteriostatic action of dyes than the other groups.

The alfalfa and bean physiological groups were chosen because they represent the extremes in resistance to hydrogen-ion concentration. According to Fred (3) the alfalfa and sweet clover cross inoculation group is inhibited in growth at pH = 4.9 and the bean group at pH = 4.2.

Several methods of applying the bacteriostatic action of the dye were studied with the idea of finding the one best suited for obtaining a pure culture.

As a check on the different methods used, the cultures obtained by the use of dyes were examined microscopically for evidence of admixture of the desired organisms with others which differ noticeably from them in morphology. To test for the absence of *B. radiobacter*, which has the same microscopic appearance as *R. leguminosarum*, the cultures which gave evidence of purity under the microscope were tested by the milk and potato tests of Löhnis and Hansen (6). These tests consist of inoculating these media with the organism to be tested and incubating for two weeks. In the case of the potato slant cultures, if the culture is a pure one of *R. leguminosarum*, the slant will have a thin slimy growth containing no pigmentation. If *B. radiobacter* is present a distinct brown coloration is produced. In the case of the milk cultures, *R. leguminosarum* produces a thin distinct serum layer on the top of the milk which is of a slimy nature and leaves the remainder of the milk unchanged in any way. In the event that *B. radiobacter* is present the milk will have the distinct serum layer on the surface, as in the case of *R. leguminosarum*, but in addition the milk portion below the serum layer will have a brown coloration.

As a check on the effect the dyes might have on the inoculating efficiency of the organisms which were isolated by this method, some of the cultures that were found to be pure were used to inoculate the species of plant from which they were obtained originally. This was done to discover the degree of nodule production they may have retained after the dye treatment.

DETAILED METHODS AND RESULTS

Technique Number One was one in which a dye agar was used. The detailed technique, in brief, was to make up relatively large amounts of the desired concentration of dye agar by adding a calculated amount of an aqueous stock solution of the dye to a flask of Ashby's agar. This was then sterilized in the autoclave at 10 pounds for 20 minutes. When the agar was removed from the sterilizer, plates were poured at once and allowed to solidify. A modification of this procedure was to add the dye to the previously sterilized agar and then pour the plates into petri dishes to allow the agar to solidify. After the agar had become solid the cultures were streaked heavily upon the surface. This technique did very little more than hold down the growth of any spreading organisms that might be present in the cultures, and even in this it was not always effective.

Technique Number Two was one making use of solutions of the desired dyes in Ashby's solution. The detailed procedure in this method was to make up the dye dilutions, with Ashby's solution, in small flasks, sterilize, and after sufficient cooling inoculate the desired cultures into the aqueous solutions of the dye. They were incubated for a week before a check was made as to their purity. The dilutions of the dye varied from $\frac{1}{100}$ to $\frac{1}{200,000}$ depending upon the dyes used. By removing a loopful of the suspension of the growing organisms from the flask and inoculating Ashby agar slants the purity of the resulting growth was determined. The results of this method were not very encouraging as it was found that very few of the dyes remained stable throughout the time of incubation. Some of the dyes decomposed very rapidly and were of no value whatever in their selective action on undesirable microörganisms. The dyes which remained relatively stable in this method also showed insufficient selective action.

Technique Number Three was one in which the probable desirable germicidal action of the different dyes was looked into. In this method 1, 0.75, 0.5, and 0.25 per cent solutions were made of the dyes to be studied. To $2\frac{1}{2}$ cc. of the dye solution in a test tube, three to five drops was added of a reasonably heavy suspension of organisms from which the desired organism was to be isolated. From this dye-organism mixture a loopful of suspension was taken after varying lengths of time; that is, a loopful of suspension was removed at the end of $\frac{1}{2}$ 1, 2, 3, 4, 5, 7, 10, $12\frac{1}{2}$, 15, 20, 30, 45 minutes, and 1 hour. A check consisting of a suspension of the organisms in sterile water was treated in the same fashion and a suspension removed at the same periods of time. This check was for the purpose of showing that any results obtained were not due to mere dilution. The loopfuls of suspension, removed at the varying intervals of time, were streaked out upon Ashby agar plates. After a week's incubation, microscopic observations for purity were made on the resulting growth, methylene blue being used to stain the smears.

The results of technique number three were considerably more encouraging than those of the preceding methods. Insufficient success was obtained to

class this method as one efficient enough for practical use. By this method it was found that those dyes which have indications of possible favorable selective action are crystal violet, brilliant green, and malachite green. Those dyes which gave some indication of selective action but did not have this characteristic strong enough to warrant their use in a more extensive study were methyl green, methyl violet, basic fuchsin, methylene blue, eosin B, eosin Y, rose bengal, and safranin. Acid fuchsin was very undesirable in its action since it actually killed the legume organisms.

In the use of this technique it was noted that considerable trouble was sometimes experienced when spores of some of the undesired organisms were present, as they withstood the germicidal action as long as the legume root nodule organisms and produced a contaminated culture. It was noted also that it was very difficult to make use of the selective action of crystal violet by this technic, for its action is very rapid in killing both the undesired and the desired organisms. Trouble arose in spacing the periods of time close enough together to check up on the action and in making dilutions which would be efficient without killing everything. In the short time (10 minutes) that the living organisms remained in contact with the crystal violet the action was noted to be decidedly favorable in that nothing but the slime-producing organisms of the root nodule remained. Brilliant green and malachite green were more controlled in their action in that they were slower in their selection of any of the organisms. The only indication of their action here was that it tended distinctly in the desired direction; that is, the legume root nodule bacteria were allowed to live longer than any "spreader"-forming organisms. No tests were madein this method on potato or milk for the presence of *B. radiobacter* because this technique was superseded by the following one.

Technique Number Four made use of the bacteriostatic nature of the dyes which were tested by this method. This characteristic of the dyes, studied in connection with the organisms in question, was found to be the best for successful isolation of the legume root nodule bacteria.

The detailed procedure of this technique was to inoculate petri dishes with a water suspension of the contents of legume root nodules and to pour over these inoculated dishes dye agar of the desired dye concentration after being cooled to a degree not uncomfortably warm to the eyelid.

The nodule suspension was obtained by the following procedure: Fair-sized nodules were selected from the plants, cut with a small pair of scissors from as much of the root as was practicable, washed as clean as possible from adhering soil, and crushed with a stirring rod in a tube of tap water. In this experiment a number of nodules were crushed in a little water in a large tube and diluted with tap water until the water was faintly turbid. The petri dishes were inoculated with a few drops of the turbid liquid by means of a pipette. The suspension was kept for some time, as it was found that the organisms remained alive in a watery suspension for a period of at least six months.

In making the dye agar, large glass test tubes were filled with enough Ash-

by's agar to make the volume after sterilization very close to 25 cc. After the agar was sterilized it either was used before it had a chance to solidify or was allowed to solidify and then stored for a few days. In the case of its immediate use the agar was cooled to a point not quite cool enough to bear on the cheek and treated with the desired dye. In case the agar had been allowed to solidify it was melted and then cooled down in the manner just described. The dye treatment consisted of adding enough of a 1 per cent aqueous-alcoholic solution of the dye to bring the concentration to the desired point. Just enough alcohol was added to the stock solution of the dye to insure complete solution and to prevent decomposition. In many cases it was found that the dyes were not very stable in water so that the alcohol was found to be very essential.

After the dye had been added to the agar and solution accomplished the agar was cooled to a point where the test tube could be comfortably held to the eyelid or cheek. When at that point the agar was poured, as has already been stated, into the previously inoculated petri dishes and allowed to solidify. After solidification the dishes were incubated at room temperature for a week and the results recorded.

The dyes which were tested by this method were crystal violet, malachite green, rosaniline hydrochloride, safranin, eosin Y, and rose bengal.

Safranin, eosin Y, and rose bengal were run with the following dilutions of dye in Ashby's Agar: $\frac{1}{500}$, $\frac{1}{750}$, $\frac{1}{1000}$, $\frac{1}{1500}$, $\frac{1}{2000}$, $\frac{1}{2500}$, $\frac{1}{3000}$, $\frac{1}{4000}$, $\frac{1}{5000}$, and $\frac{1}{10,000}$. Above $\frac{1}{1000}$ there was little bacteriostatic effect on bacterial species and none of the concentrations used restrained the growth of molds. On the whole these dyes were too weak to be useful and they were eliminated after one trial.

Rosaniline hydrochloride was run at the following dilutions: $\frac{1}{100}$, $\frac{1}{200}$, $\frac{1}{300}$, $\frac{1}{400}$, $\frac{1}{500}$, $\frac{1}{750}$, $\frac{1}{1000}$, $\frac{1}{10,000}$, and $\frac{1}{20,000}$. This dye also allowed the growth of molds to too great an extent for the satisfactory isolation of *R. leguminosarum*. At the $\frac{1}{100}$ dilution of the dye the alfalfa strains showed no growth and the bean strains a few colonies of a nature characteristic of the legume root-inoculating bacteria. This dilution failed to show any tendency to retard the growth of molds since the dye medium had as heavy a crop of mold growth as did the check plates without dye. The mold which appeared was not positively identified but was either Rhizopus of Mucor and proved very bothersome in making the final isolation of colonies in a pure condition. The bacterial colonies which developed in the bean nodule plates in this dilution ($\frac{1}{100}$) were all pure and appeared to be *R. leguminosarum*, as judged by observing the colonies with the naked eye. At $\frac{1}{200}$, colonies put in their appearance on the alfalfa nodule plates and the number on the bean nodule plates increased accordingly. The $\frac{1}{300}$ plates gave much the same results as did the $\frac{1}{200}$, and in the $\frac{1}{400}$ and up to $\frac{1}{2000}$ other bacterial growths began to appear. Under the microscope the cultures obtained from the $\frac{1}{100}$, $\frac{1}{200}$, and $\frac{1}{300}$ plates all upheld the evidence pointed out by the macroscopic observations of the plates;

that is, colonies of the legume root-inoculating organisms could be obtained in absolute purity. No tests were made from the cultures obtained by the use of this dye for *B. radiobacter*, because molds were so troublesome when this dye was used. For this reason the dye was considered insufficient for our purpose.

Malachite green gave considerably more encouraging results than rosaniline hydrochloride in its selective reactions. With this dye, in the first series of tests, dilutions of $\frac{1}{10000}$, $\frac{1}{15000}$, $\frac{1}{20000}$, $\frac{1}{25000}$, $\frac{1}{30000}$, $\frac{1}{40000}$, $\frac{1}{50000}$, $\frac{1}{75000}$, $\frac{1}{100000}$, and $\frac{1}{200000}$ were made and it was found that no dependable growth of any sort would be allowed by this dye at any dilution from $\frac{1}{10000}$ to $\frac{1}{25000}$, for no growth whatever was observed up to that point in the alfalfa nodule cultures and only a few colonies on the $\frac{1}{15000}$ plates in the case of the bean nodule culture (probably due to improver mixing of the dye at that particular dilution). From $\frac{1}{30000}$ on, the number of colonies of *R. leguminosarum*-like nature increased and continued to be the only organisms on the dye plates up to a dilution of $\frac{1}{75000}$. Molds were inhibited by this dye. On the plates with dye dilutions of $\frac{1}{100000}$ and up, the purity of the colonies was a matter of some question. All secondary experiments with this dye, after the first series was made, were run with dilutions ranging from $\frac{1}{20000}$ up to $\frac{1}{75000}$. A dilution of $\frac{1}{40000}$ or $\frac{1}{50000}$ was found to allow the growth of a fair representation of the desired type of colonies, which under the microscope were found to be pure cultures of what appeared to be the desired organisms, and yet prevented the growth of any other types of bacteria. Cultures of the colonies on the two types of plates—alfalfa and bean—were saved from this dye and run for purity on milk and potato, as has been mentioned before, and the purity was found to be rather inconsistent and undependable at any concentration.

The dye which gave the most decided and favorable results was crystal violet. Dilutions of $\frac{1}{50000}$, $\frac{1}{75000}$, $\frac{1}{100000}$, $\frac{1}{150000}$, $\frac{1}{200000}$, $\frac{1}{250000}$, and $\frac{1}{300000}$ were made for the first experiment with this dye and dilutions of $\frac{1}{75000}$, $\frac{1}{100000}$, $\frac{1}{150000}$, $\frac{1}{200000}$ and $\frac{1}{250000}$ for all the subsequent experiments. With this dye, as in the others, it was found that the alfalfa strain showed a slightly weaker tendency to recover from the effects of the dye than did the bean strain, for at the $\frac{1}{50000}$ dilution the bean strain gave several medium-sized colonies, whereas the alfalfa strain had but one colony. With the $\frac{1}{75000}$ dilution the alfalfa strain colonies began to show up, and at a dilution of $\frac{1}{100000}$ one could depend on a fair representation of colonies of the organisms having the characteristics of *R. leguminosarum*. Dilutions of $\frac{1}{100000}$ and $\frac{1}{150000}$ were thought to be the most satisfactory, as at these dilutions colonies in sufficient numbers and of sufficient size were obtained in every test and yet the concentration was such as to prevent the appearance of colonies of any other type. At $\frac{1}{250000}$ a few colonies of gram-negative organisms of other types were noted. No molds appeared on any of the plates in which this dye was used; this proved of some distnct advantage in the picking of the colonies.

Cultures were obtained from colonies which grew on the plates with the dilutions of $\frac{1}{50000}$, $\frac{1}{75000}$, $\frac{1}{100000}$ $\frac{1}{150000}$, $\frac{1}{200000}$, and $\frac{1}{250000}$ of crystal violet. These

cultures were all tested by the method of Löhnis and Hansen (6) for freedom from *B. radiobacter*, as already described in this paper. They were also tested for inoculating efficiency, as has already been stated. The purity test on potato and milk showed all the cultures obtained by this dye up to and including those obtained from a dilution of $\frac{1}{125000}$ to be free from *B. radiobacter*. The $\frac{1}{200000}$ dilution culture and those from higher dilutions gave very irregular results in the matter of freedom from *B. radiobacter*, so that it would appear that in these dilutions this contaminant was not eliminated.

The cultures obtained from the crystal violet plates with dye dilutions of $\frac{1}{50000}$ and $\frac{1}{75000}$ for both the alfalfa and bean strains were tested for their inoculating efficiency by either growing the plants in question in a large test tube of Ashby's agar or in sterile sand inoculated with the strain of the organism isolated by the dye method. In either event the plants were allowed to grow for five or six weeks in the presence of the organisms and then observed for nodules. In the case of the alfalfa strain isolated from the $\frac{1}{50000}$ dye dilution, the test tube method was used; all other tests were made in sterile sand which had been inoculated with the strain of the organism to be tested. In the tube of Ashby's agar, in which the alfalfa plant was grown in company with the culture isolated from the $\frac{1}{50000}$ dye agar plate, a heavy crop of nodules was noted, showing that at that concentration the efficiency of the strain was not in any way weakened. Moreover, on all the other tests for efficiency of the strains isolated through the use of crystal violet, nodules were formed in very satisfactory amounts, which gives further evidence that the organisms survive the severe treatment to which they are subjected during the contact with the dye.

A presumably pure culture of *B. radiobacter* was treated by the crystal violet method of isolating the legume-inoculating bacteria and colonies of a characteristic nature were obtained in dilutions, even below $\frac{1}{200000}$. These strains were then tested for purity by the means already described, and it was found that *B. radiobacter* was absent. They were at the same time used to inoculate a number of alfalfa and bean plants and found to induce a good crop of nodules of the alfalfa roots. This was undoubtedly due to the culture of *B. radiobacter* being mixed with alfalfa-inoculating bacteria. Before subjecting it to the crystal violet treatment the culture was inoculated into milk and found to give the brown coloration, showing that *B. radiobacter* was present.

As a final test of the practicability and simplicity of this method of isolating *R. leguminosarum* by the aid of dyes, an exercise was made up for a class in beginning bacteriology and submitted to them for trial. The class was uniformly successful and obtained pure cultures of *R. leguminosarum* at the very first trial. The dilutions suggested for the use of the class were $\frac{1}{100000}$ and $\frac{1}{125000}$. A few were asked, as a demonstration of the extremes of the original experiment, to run dilutions of $\frac{1}{75000}$ and $\frac{1}{200000}$. The findings in the "side" experiments were similar to those obtained by the writer. No tests for purity were made by the method of potato or milk inoculations on the cultures obtained by the class.

CONCLUSIONS

From the foregoing results, which were repeated several times, it is reasonable to conclude:

1. That subjecting *R. leguminosarum* to severe treatment with dyes does not in any way affect the ability of the legume root nodule-producing bacteria to produce nodules on the species of plants from which they were originally isolated. This was arrived at by observing that cultures recovered from dye plates which almost entirely inhibited all growth were still able to produce nodules on the plants.

2. That such a method of isolating *R. leguminosarum* is possible and practicable through the use of crystal violet dissolved in Ashby's agar in concentrations of 1 part of dye to 10,000 of medium, down to 1 part of dye to 15,000 parts of medium. This method was found so simple and convenient that beginning students in bacteriology could get successful results in their first trials.

3. Other dyes which gave promising results, although not so clear-cut as the results with crystal violet, were rosaniline hydrochloride and malachite green.

4. The foregoing results were confined to nodules from locally grown alfalfa and bean plants. Time did not permit of extending these studies to the nodules of other leguminous plants nor to plants from other parts of the country.

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CONTRIBUTION TO THE CHEMICAL COMPOSITION OF PEAT: V. THE RÔLE OF MICROÖRGANISMS IN PEAT FORMATION AND DECOMPOSITION[1]

SELMAN A. WAKSMAN AND KENNETH R. STEVENS

New Jersey Agricultural Experiment Station

Received for publication April 5, 1929

Microörganisms in general play four distinct parts in the chemical transformations leading to peat formation and decomposition:

1. Those microörganisms which are active during the first stages of decomposition of the plant residues, either before or after the residues have become submerged with water; in these processes of disintegration some of the chemical constituents of the plants rapidly disappear. Certain groups of organisms, largely fungi on the surface of the bog and bacteria below the surface of the anaerobic medium, bring about the decomposition of the sugars, of certain hemicelluloses, of the celluloses, and of some proteins and their derivatives. The carbon dioxide and ammonia thereby liberated are used immediately by the growing plants; this process is of great importance in bogs poor in nutrients, like the highmoor peats.

2. Microörganisms active in the various horizons of the peat profile, long after the initial stages of decomposition have passed. Here we are dealing almost entirely with facultative and obligate anaerobic bacteria. The pockets of gas rich in hydrogen and methane, as well as certain putrefactive odors, frequently found at various depths of the peat profile are due to the gradual decomposition of the celluloses, of the proteins, and of other complexes by these bacteria.

3. Microörganisms active in the decomposition of the organic complexes in the peat, after the bog is drained, in the case of lowmoor and sedimentary peats, or drained and limed, in the case of the highmoor peats. Here we are dealing with various fungi, aerobic bacteria, and actinomyces decomposing the resistant peat complexes with the liberation of large quantities of ammonia, which is rapidly changed to nitrates by nitrifying bacteria. These nitrates may accumulate in quite considerable quantities in the surface layers of the drained peat. Frequently and under certain conditions, especially in highmoor bogs receiving excessive amounts of lime, nitrate reducing bacteria may become active; this leads to losses of nitrogen to the atmosphere.

4. Microörganisms that have contributed directly by their cell substance to the formation of certain peats. This is true especially of sedimentary or allochtonous peats in which fungus spores and mycelium, as well as various algae and bacteria, may be quite abundant.

Peat is formed because the saturation of the bog with water produces anaerobic conditions. This does not prevent the growth of plants adapted to that environment, but it does prevent the growth of fungi, actinomyces, and aerobic bacteria which would be capable of decomposing the plant residues. The obligate and facultative anaerobic bacteria favored by these conditions are capable of attacking only some of the organic complexes, leaving the other

[1] Journal Series paper of the New Jersey Agricultural Experiment Station, Department of Soil Chemistry and Bacteriology.

constituents to accumulate, and thus give origin to peat. The lignins, either as such or in a modified form, certain nitrogenous substances (proteins, etc.), and various hemicelluloses predominate in lowmoor peats; whereas waxes, certain celluloses and hemicelluloses, and lignin-like complexes predominate in the highmoor peat formations.

No other phase of peat investigation has been more confused and no other phase suffers from a greater lack of accurate information than the proper understanding of the rôle played by microörganisms in peat formation and in peat transformation. Some chemists deny altogether the fact that microbes take any part in the transformation of plant substances into peat; they either consider the process as one of spontaneous decomposition of plant material, giving origin to peat, or believe that the transformations are a result of the action of atmospheric agencies, as those of oxidation and reduction.

Potonié (29), for example, considers the process of peat formation as one of "self decomposition," which represents the last phase of oxidation, decomposition, and fermentation processes. According to Früh (13), peat formation is not a result of bacterial action, but consists in a slow breakdown, at low temperatures and in the absence of oxygen, of the plant constituents, with an inner oxidation, resulting in the liberation of water:

$$C_6H_{10}O_5 \rightarrow C_6H_8O_4 \rightarrow C_6H_6O_3 \rightarrow C_6H_2O$$

He states quite specifically that the process of peat formation is not one of fermentation nor is it one of bacterial action, but consists in a slow decomposition of plants in the complete absence of oxygen, due to the presence of water and at low temperatures, but, he adds, "Spaltpilze haben mit der Torfbildung nichts zu tun."

Similar ideas have been expressed by Kauko (20) and others. Oden (27) also speaks of the rôle of atmospheric agencies in peat formation. Some of the chemists consider the whole problem of peat formation in even simpler terms: according to these conceptions, the carbohydrates which are formed from the decomposition of the celluloses and the amino acids produced from the proteins combine at high temperatures to give dark colored substances (25). The fact that neither sugars are formed nor do amino acids accumulate under the natural soil and bog conditions, as well as the fact that the required high temperature will not be attained under such conditions, do not matter much, as long as the logic is correct.

Kürschner (22) is, therefore, quite justified in speaking that all the processes termed "self-decomposition," "breakdown," "autolysis," etc. are merely attempts to explain x by y, or one unknown factor by another equally unknown without any attempt to get a deeper insight into the problem.

An understanding of the functions of microörganisms in peat formation necessitates a knowledge of the nature of the organisms commonly found in the various horizons in the natural peat profile, their activities, and their rôle in peat transformation. An attempt will be made in this paper to sum-

marize our present information and to throw further light upon this complicated problem.

HISTORICAL

As mentioned above, Früh (13) was among the very first to report in 1883 that peat bogs are free from bacteria, i.e. are practically sterile. However, the bacteriologist Gaffky (14) claimed that bacteria are present in peat bogs. The well-known peat investigator, Weber, stated in 1890 that bacteria are found only in the surface layers of peat. Both Proskauer (30) and Benni (5) soon demonstrated that bacteria may be found even in the lower depths of peat bogs.

Ramann and associates (31) found bacteria in great abundance in the upper layers (0 to 5 cm. depth) of young peat formations, the numbers ranging from 200,000 to 2,710,000 cells of bacteria per gram of dry material; the existence of these organisms at lower depths was doubted, however. Stalström (36), as well, found that all peat samples taken from a depth of 50 cm. and lower were sterile; he claimed, therefore, that the presence of microörganisms is limited to the upper 20 cm. of the bog.

Fabricius and von Feilitzen (10) reported the presence of 56,000 to 225,000 bacteria per gram of moist peat taken at a depth of 35 cm. Fischer (11) found as many as 1,000,000 bacteria in one gram of well decomposed peat and 950,000 in young peat. Ritter (32) found fewer bacteria in young that in old peat; he rarely encountered peat entirely free from bacteria. Even assuming the existence of bacteria in peat bogs, Wehmer (45) still doubted whether the activities of these organisms could fully explain the problem of "humin formation."

All the aforementioned investigators used the aerobic plate method (with meat peptone agar or gelatin, or peat extract agar) for determining the abundance of bacteria in peat. Their results point to the existence in natural peat of very small numbers of bacteria capable of developing under these conditions. These numbers were at best only about one-tenth of the numbers of bacteria commonly found in field soils by the use of the same method. It is doubtful whether the methods used for determining the presence and abundance of microörganisms in peat were satisfactory in most of the aforecited studies. Keppeler (21) has already expressed his grave doubts of the accuracy of those results, because of difficulties involved in experiments of this nature.

Begak (4) recently made a study of the distribution of bacteria in various profiles of a sphagnum peat, using meat-peptone agar as a nutrient medium. The numbers were found to be around 4,000 cells per gram of peat in the upper 10 cm. of the bog, increasing to 90,300 at 25 to 30 cm., then diminishing with depth, so that, at 2.0–2.5 meters depth (at the Grenzhorizont), there were only 2,800 cells per gram; below that layer, the numbers increased again to 9,400 cells per gram of peat. The numbers of bacteria in the waters of the dead sphagnum and in the sphagnum itself were constant throughout the summer,

namely, about 4,000 cells per gram. About eight times as many bacteria were found in the well-decomposed layers of the peat as in those that were only partly decomposed. It is interesting to note that the abundance of organisms found by Begak, by the use of the plate method, in the Grenzhorizont was later confirmed by microscopic examinations; the author noted thereby also a very abundant flora of fungi forming very minute colonies.

By the use of the direct microscopic method of soil examination (after Winogradsky), Begak found 323 to 715 millions of bacteria per gram of moist sphagnum, which would give three to seven billion cells in each gram of dry sphagnum. These bacteria were made up of 1.25 per cent large rods, 60.0 per cent short rods, 20 per cent cocci, and about 20 per cent thick rods and small cocci. This abundant microflora of bacteria as well as an abundant development of fungi were believed to prove that these are the active agents in the decomposition processes taking place in the peat. The acidity of the sphagnum was equivalent to pH 3.3 to 4.2. The bacteria present there were found to be adapted to this high acidity.

Ritter (32) previously criticized the Koch plate method as altogether unsuitable for giving any idea concerning the abundance of the bacterial population in peats. The nitrifying, anaerobic, nitrogen-fixing, and other organisms do not develop at all on this medium. The colloidal nature of the peat makes an even distribution of the bacteria even more difficult. His results in general showed that the cell content of uncultivated highmoor peats is usually very low; young or little decomposed peat is always poorer in bacterial cells than well-decomposed peat. Peat cultivated for a long time, limed and fertilized, was found to contain incomparably more bacteria than untreated peat; when peat is well decomposed, it may contain more bacteria than mineral soils. An untreated peat receiving only an application of lime showed a relatively moderate increase in the bacterial cells. Lowmoor peats contained in all cases more bacteria than highmoor peats, even in an uncultivated state. As in mineral soils, the numbers of bacteria were found to decrease rapidly with depth, but they were still present even at a depth of 50 cm., when infection was fully excluded. A difference in the composition of the medium could not show any great variation in the numbers of bacteria. It may be of interest to call attention, in this connection, also to the results of White and Thiessen (46), who reported the presence of anaerobic bacteria in peat bogs even at a depth of 9m.

The nature of the peat was found to exert a marked influence upon the nature and abundance of the bacterial population. Stalström (36) found more bacteria in lowmoor peats than in highmoor peats; drainage brought about a great increase in bacterial development, especially when the peat is mixed with clay or manure. Fabricius and von Feilitzen (10) explained the low bacterial content of highmoor peats by the high acidity; drainage alone did not bring about any marked bacterial multiplication; liming, cultivation, manuring, and treatment with sand brought about considerable development

of bacteria. Well-manured and cultivated highmoor peat soil, however, contained as many bacteria as lowmoor peat soil under the same conditions. Soil temperature was also found to influence appreciably bacterial development. These results can be summarized as follows:

	Numbers of bacteria per gram, by the plate method
Raw, uncultivated peat	138,500
Drained, but not cultivated peat	200,300
Freshly cultivated, highmoor peat treated with sand and lime	6,900,400
Long cultivated highmoor peat treated with sand, lime, and manure	6,224,500
Same, under fallow	7,801,000
Same, with oats grown	7,175,000

Arnd (1) also found that the addition of lime to highmoor peat greatly stimulates the development of various groups of bacteria, especially the ammonia-forming organisms, although these are present abundantly in natural peat bogs of different types; the numbers and activities of these organisms in the deeper layers are considerably less than in the upper layers. Drainage, cultivation, and liming of highmoor peats were all found (34) to have a marked effect in increasing the numbers and activities of various groups of microorganisms, largely in the surface layers of peat.

It has been shown repeatedly that nitrifying bacteria are absent in highmoor peat bogs, although Chouard (7) observed that nitrification takes place even in certain acid peats. When a highmoor peat is moderately limed, no nitrification will take place within the first year; an excessive addition of lime will bring about rapid development of nitrifying bacteria. A moderate amount of lime will increase crop productivity, but an excess of lime above certain limits will not result in such high crop yields. This was explained (23) by the fact that the increase in the amount of lime added to the peat stimulates the development of denitrifying bacteria, organisms which may bring about considerable losses of nitrogen (16).

Azotobacter is absent in highmoor peat, while *Bac. amylobacter* is present both in cultivated and in uncultivated peat. Leguminous plants are totally absent in highmoor peats. In lowmoor peats nitrite and nitrate forming bacteria are active. Cellulose decomposing bacteria are abundant in the lowmoor peats, especially when soluble P_2O_5 is added. For the development of these bacteria in highmoor peats, the addition of calcium carbonate is essential.

According to Tacke (38), the addition of stable manure to peat has a favorable effect in stimulating the liberation of nutrients. The activities of the microörganisms were measured by adding 500 mgm. of nitrogen in the form of peat to 500 gm. of sandy soil; this was adjusted to optimum (16 per cent) moisture content and incubated for 70 days at 22°. Manured peat liberated 20.5–22.2 mgm. of nitrogen as nitrate, whereas unmanured peat liberated only 6.3 mgm. of nitrate nitrogen. The favorable action of manure was ascribed to the stimulating effect upon the activities of microörganisms in the peat.

To bring a highmoor peat into a condition when it can grow agricultural crops profitably, it has to be first drained, then fertilized with phosphorus, potassium, and, in the absence of leguminous plants, with nitrogen, and finally limed. These treatments modify considerably the activities of microörganisms, both qualitatively and quantitatively. Drainage permits the aeration of the peat material, penetration of oxygen thus enabling the aerobic organisms to develop; it produces a change from an anaerobic to an aerobic system. The removal of the water allows the soil to become warmed, thus influencing favorably the activities of microörganisms. Liming of a highmoor soil creates favorable conditions for the activities of many organisms. Arnd believed that even fungi may thus be favorably affected.

Although the Remy solution method was used by Arnd (1) for comparing the activities of microörganisms in different layers of a highmoor peat under different treatments, a method not very satisfactory for measuring the activities of the microbial population in normal field soils, marked differences were obtained: surface material from an untreated peat bog produced, in six days, from a 50-cc. peptone solution, 3.1 mgm. of nitrogen as ammonia, while peat from a depth of 20–40 cm. produced only 0.8 mgm. nitrogen as ammonia. The same peat, when drained, limed, and fertilized produced 8.2–10.3 mgm. of ammonia nitrogen; when drained, limed, fertilized, and manured peat was used, 27.8 mgm. of ammonia nitrogen was liberated.

In general, highmoor peats were found to be considerably less active biologically than lowmoor peats. Christensen (6) characterized these two types of peat microbiologically as follows:

> Highmoor peats possess a low peptone-decomposing capacity, no nitrifying power, considerable denitrifying capacity, and a very limited microbial population capable of decomposing celluloses and mannitol. Lowmoor peats have a strong peptone-decomposing, nitrifying, denitrifying, and mannitol-decomposing capacity but a low cellulose-decomposing power. In the latter respect different peats may vary considerably.

Lowmoor peats were found to contain, at the surface and to some depth as well, a considerable number of actinomyces. The number of these organisms increases especially when the bog is drained and cultivated. It is believed that the actinomyces take an active part in the decomposition of some of the resistant complexes in the peat and lead to the liberation of the nitrogen in an available form.

Lowmoor peats have a vigorous flora of nitrifying and cellulose-decomposing bacteria. These are largely responsible for the abundant formation of nitrates in the upper layers of the peat and the almost complete lack of celluloses among the chemical peat constituents. When a lowmoor peat is moistened with ammoniacal solution, it forms a very excellent medium for the formation of nitrates, provided the compost is properly aerated and the reaction is not allowed to become too acid. Fungi are found to be abundant in lowmoor peats, but only at the very surface of the undrained bog.

Highmoor peats, however, because of their high acidity are free from nitrifying and aerobic cellulose-decomposing bacteria as well as actinomyces. They contain a highly specific bacterial flora, partly aerobic, partly facultative anaerobic, and partly obligate anaerobic, which grows readily at pH 4.0, a phenomenon not observed commonly among soil bacteria. A large number of these acid-resisting bacteria have been isolated from the various depths of the Maine profiles and a detailed description of these will follow later.

Omeliansky (28) found in lake mud or sedimentary peat formations the presence of the following groups of bacteria: proteolytic, denitrifying, anaerobic nitrogen-fixing, pectin-fermenting, aerobic cellulose-decomposing, anaerobic cellulose-decomposing, and fat splitting. He came to the conclusion that sedimentary peat (Sapropel) represents a medium in which various bacterial processes take place energetically, leading to the decomposition of proteins, carbohydrates, and fats.

As to the animal population of peat bogs, we find that an abundant fauna of rhizopods has been recorded to be present in sphagnum peats, some of the organisms preferring this habitat (18, 17). Distinct differences were observed between different types of sphagnum (37). Fully developed highmoors possess a definite, frequently abundant, flora of *Amphitrema* species (*flavum* and *wrightianum*). These may be missing in non-fully developed peats, but *Hyalosphenia papilio* and *H. elegans* are common to both. Highmoor peats contain also considerable numbers of protozoa not commonly found on sphagnum itself.

A study of a number of samples of sphagnum peat revealed (17) the following groups of Rhizopoda: (*a*) Species of *Difflugia*, *Centropyxis*, *Arcella*, *Nebela*, *Euglypha*, *Assulina*, *Corythion*, and *Trinema*. This group is recognized as belonging to the "forest-moss type," although distinctly sphagnum types, such as *Nebela militanis*, are also found here. (*b*) Some species of group *a* and, in addition, *Hyalosphenia papilio* and *H. elegans;* a more constant and regular fauna, known as the "*Hyalosphenia* type." (*c*) In addition to the above, *Amphitrema* species are present; fauna of this type is richest in kinds and is known as the "*Amphitrema* type." (*d*) *Quadrula symmetrica* predominates, also *Cyphoderia* and *Nebela* species. In general, the types of rhizopod associations found in peat bogs correspond to definite botanico-geological peat types. The abundance of fossil rhizopods in peat was pointed out by Lagerheim (23) and others. Many of these species are found in a fossil condition in the various layers of peat in quite considerable numbers.

The great abundance of algae in certain peat formations, frequently even forming special types of peat, has been established by various investigators (15) and need not be discussed here. The presence in peat of a considerable fauna of nematodes, oligochaetes, myriapods, insects, and various other invertebrates has also been established (17).

EXPERIMENTAL

Occurrence of microörganisms in natural peat formations

The nature and chemical composition of the peats used in the following investigations have been described in detail previously (41). Two types of lowmoor peat profiles, one from New Jersey (41) and one from the Everglades, Florida (42), and two highmoor peat profiles from Maine (43) were used for this purpose.[2] In the case of the New Jersey and Maine peats, samples were taken by the authors into sterile glass containers and analyses immediately made when the samples arrived at the laboratory.

A synthetic medium (egg-albumin agar) was employed in determining the numbers of bacteria by the plate method; the abundance of anaerobic bacteria

TABLE 1

Occurrence of microörganisms at different depths of a lowmoor peat profile from Newton, N. J.

On the basis of fresh peat material

DEPTH OF SAMPLE	pH	MOISTURE CONTENT	BACTERIA (AEROBIC AND FACULTATIVE AEROBIC AND) ACTINOMYCES	ACTINOMYCES	FUNGI	AEROBIC CELLULOSE DECOMPOSING BACTERIA*	NITRIFYING BACTERIA*	ANAEROBIC BACTERIA*
cm.		*per cent*		*per cent*				
Surface	5.9	61.1	6,000,000	90	105,000	++	+++	+
30	6.0	72.5	350,000	40	250	+	++	++
45	6.2	82.3	450,000	25	175	0	++	++
60	6.3	87.5	40,000	20	150	0	+	++
75	6.3	87.1	35,000	25	33	0	+	++
90	6.4	80.8	20,000	15	0	0	0	++
120	6.7	83.6	100,000	2	0	0	0	+++
150	6.8	84.5	500,000	0	0	0	0	++++
165	8.0	64.8	200,000	0	0	0	0	++++
Clay bottom								

* + designates a few; ++ a fair number; +++ abundance of organisms; ++++ numerous (about 25,000 or more colonies formed by 1 gm. of material).

was measured by the use of the same medium in a shake tube. For demonstrating the presence of cellulose-decomposing bacteria, tubes with liquid medium containing strips of cellulose as the only source of energy were found to be quite convenient (9).

Tables 1, 2, 3, and 4 give the numbers of aerobic and facultative anaerobic bacteria, per cent of actinomyces, numbers of fungi, abundance of anaerobic

[2] The samples from the Everglade profile were taken under sterile conditions by Dr. R. V. Allison of the Belle Glade Station (Florida), to whom the authors are indebted for this courtesy. The authors wish to acknowledge here again their indebtedness to Dr. A. P. Dachnowski-Stokes, of Washington, D. C., for coöperation in the taking of the samples from the Maine profiles.

bacteria, cellulose-decomposing and nitrifying bacteria in 1-gm. portions of fresh peat taken at different depths of the different profiles.

The results of the microbiological analysis of the lowmoor peat profile from Newton, N. J., show an abundant development of bacteria throughout the whole profile, from the surface to the underlying clay. The greatest numbers of aerobic and facultative aerobic bacteria occur at the very surface and diminish rapidly with depth. Below a certain depth, namely, at about 90 cm., however, the numbers of bacteria begin to increase, largely because of the increase of anaerobic forms. This is well brought out in the results of the shake tube method, where the anaerobic bacteria are found to increase rapidly with depth. These tubes were prepared from a dilution of 1 to 50; numerous colonies of anaerobic bacteria were found in the tubes prepared from the lower parts of the profile. Among these bacteria the butyric acid organisms were quite abundant. It is to be recalled in this connection that only a small proportion of the viable anaerobic bacteria are capable of developing into colonies.

TABLE 2

Occurrence of microörganisms at different depths of a lowmoor peat profile from the Everglades, Fla.

On the basis of fresh peat material

DEPTH OF SAMPLE	pH	MOISTURE	BACTERIA (AEROBIC AND FACULTATIVE AEROBIC AND ACTINOMYCES	ACTINOMYCES	FUNGI	CELLULOSE DECOMPOSING BACTERIA	NITRIFYING BACTERIA	ANAEROBIC BACTERIA
cm.		*per cent*		*per cent*				
Surface	6.2	66.8	9,600,000	35	26,000	++	+++	+
26–40	6.4	71.4	32,800,000	22	2,000	++	++	++
50–62	6.5	85.0	3,000,000	23	0	++	++	++
110–120	6.3	83.4	1,600,000	0	0	+	+	+++

The actinomyces and fungi are very numerous at the surface of the peat and diminish rapidly with depth. The same is true of the nitrifying and aerobic cellulose-decomposing bacteria. It is interesting to note that the rapid drop in the abundance of nitrifying and cellulose-decomposing bacteria, fungi, and actinomyces was quite parallel with the drop in the total numbers of bacteria as determined by the plate method. On the other hand, the increase in the number of bacteria at a depth below 90 cm. was accompanied by an increase in the abundance of anaerobic bacteria, by a total lack of aerobic cellulose-decomposing and nitrifying bacteria, by the disappearance of fungi, and by a rapid drop in the numbers of actinomyces.

An attempt was also made to determine the abundance of *Bact. radiobacter* cells, by plating out the peat on glycerine-nitrate soil extract agar containing crystal violet, in concentration of 1 gm. of dye to 100,000 parts of medium. About 275,000 cells of *Bact. radiobacter* were found per gram of fresh surface peat. The numbers then diminished rapidly with depth and disappeared altogether at 90 cm.

These results indicate that from the surface to a depth of 90 cm. an aerobic flora prevailed in this particular peat formation, although it diminished rapidly below the surface few centimeters. At depths lower than 90 cm., the aerobic

TABLE 3

Occurrence of microörganisms at different depths of a highmoor peat profile from Cherryfield, Me.

On the basis of fresh material

DEPTH OF SAMPLE	pH	MOISTURE CONTENT	AEROBIC AND FACULTATIVE AEROBIC BACTERIA PER GRAM	ACID-RESISTING ANAEROBIC BACTERIA
cm.		*per cent*		
Surface layer			250,000	0*
7.5–20	4.05	92.7	100,000	+
20–30	3.95	92.6	220,000	+
30–45	3.85	92.6	1,600,000	+
45–60	3.86	92.9	3,500,000	++
60–75	3.73	93.6	1,500,000	++
120–150	3.90	93.6	2,100,000	+++
175–210	4.47	93.4	750,000	++
450–480	4.71	92.4	800,000	+++
540–570	5.18	92.2	2,000,000	++++

* The determinations of the numbers of anaerobic bacteria were carefully repeated using dilutions prepared so as to enable a more accurate counting of the organisms; 15,000 cells of bacteria capable of developing into colonies were found in each gram of moist peat in the 20–30 cm. layer, 550,000 in the 45–60 cm. layer, and 350,000 in the 120–150 cm. layer.

TABLE 4

Occurrence of microörganisms at different depths of a sphagnum peat profile from Orono, Me.

On the basis of fresh material

DEPTH OF SAMPLE	pH	MOISTURE CONTENT	AEROBIC AND FACULTATIVE ANAEROBIC BACTERIA PER GRAM	ACID-RESISTING ANAEROBIC BACTERIA
cm.		*per cent*		
2.5–10	4.35	94.2	100,000	++*
15–20	4.30	93.9	120,000	+
22.5–30	3.95	91.8	260,000	+
90–120	4.13	93.7	650,000	++
150–180	4.20	95.0	750,000	+++
240–270	5.70	92.6	1,250,000	++
270–330	6.04	89.9	2,000,000	+++

* Repeated determinations of the number of anaerobic bacteria, by the shake tube method, using only two samples taken at the 15–20 cm. and at the 90–120 cm. levels, gave 180,000 and 190,000 cells capable of developing into colonies per gram of moist peat.

flora disappeared completely and was replaced by an anaerobic flora. Attention should be called here to the fact that this bog underwent a certain amount of draining, as shown by the comparatively low moisture content of the peat within the upper 45 cm.

The results of the microbiological analysis of the lowmoor peat profile from Florida (table 2) are very similar to the results obtained in the analysis of the Newton profile, with certain minor differences. Here as well, one finds a very extensive bacterial flora from the surface of the bog to a depth of 40 cm. Below this layer the numbers, as shown by their development on the ordinary standard agar plate, diminish. The actinomyces, fungi, and aerobic cellulose-decomposing and nitrifying bacteria also diminish with depth, and their greatest abundance coincides with the greatest development of aerobic bacteria as determined by the plate method. The anaerobic bacteria, in this peat as well, increase with depth.

The occurrence and abundance of microörganisms in the sphagnum peat profiles present a distinctly different picture from that found in the two lowmoor peats. The sphagnum peats, being very acid in reaction, would naturally not be expected to contain many, if any at all, of certain types of bacteria; one would not expect to find, for example, organisms like the cellulose-decomposing bacteria, the nitrifying bacteria, and the nitrogen-fixing *Azotobacter*, which are known (40) to have a lower acid limit (higher pH value) than that found in these peats. A review of the previous literature on the occurrence of bacteria in natural highmoor peat formations actually points to the absence of *Azotobacter* and of nitrifying bacteria in these peats. The results of a microbiological study of the sphagnum peat profiles from Maine fully confirm those observations (tables 3 and 4). The nitrifying and aerobic cellulose-decomposing bacteria were found to be lacking altogether. The fungi were present at the very surface of the bog but not below the surface layer. Actinomyces were also lacking almost entirely. The bacteria developing on the plate were largely anaerobic or facultative anaerobic in nature. Contrary to the general expectations, the numbers were found to increase rapidly with depth, as shown both by the plate and the shake tube method. Most of the bacteria were acid resistant as shown by their growth in a medium (11) of pH 4.0, which was used for determining the numbers of these organisms by the shake tube method. The anaerobic bacteria developed in this medium very abundantly, producing gas within 20 to 48 hours. Hundreds of colonies were found in each tube of dilutions of 50 to 500 made of the peat taken even from the lowest depth of the profile. Among these anaerobic organisms, butyric acids and alcohol-forming bacteria were found to occupy a prominent place. However, cellulose-decomposing bacteria were lacking almost entirely or were found only in a few isolated instances. Most of the bacteria found in the lower layers were capable of using only sugars and proteins as sources of energy.

Quite similar results were obtained from two other sphagnum profiles investigated. This proves conclusively not only the existence of an abundant bacterial flora in the whole sphagnum peat profile, but also that this flora is quite specific, depending on the nature of the peat and upon the composition and depth of the particular horizon.

Decomposition of peat by microörganisms

The rôle of bacteria in the slow but gradual transformation of peat which has been laid down many years ago is still a matter of dispute. Some investigators claim (29, 39) that anaerobic bacteria are constantly at work decomposing the proteins and celluloses of the peat; others (19) deny entirely any possible action of bacteria and fungi, once the organic residues have been laid down under water. This may be either because of the lack of proper organisms, the unfavorable conditions under which the decomposition processes have to take place, or the resistance of the particular plant residues to attack by those organism which are capable of living under the particular conditions. Melin et al (26) found, for example, that when peat is allowed to undergo a process of fermentation, using sewage sludge as an inoculum, only the celluloses and hemicelluloses are decomposed but not the lignins or the so-called humic acids.

From the point of view of practical utilization of peat, the decomposition processes which result in the liberation of the constituent elements, especially of the nitrogen and carbon, in forms available for plant growth are of prime importance. The evolution of carbon dioxide is a good index for measuring the rapidity of decomposition of peat material, while the formation and accumulation of ammonia and nitrate can be used for measuring the rapidity of liberation of the nitrogen in an available form.

It has been shown elsewhere (44) that in the decomposition of grasses and cereal straw, as well as of sedges and reeds, none of the nitrogen in the plant is made available during the early stages of decomposition. As a matter of fact a considerable amount of additional combined inorganic nitrogen may be required for a rapid disintegration of the plant materials to take place. This is because the bacteria and fungi bringing about the decomposition of the celluloses and of the hemicelluloses consume large quantities of nitrogen for the synthesis of their cell substance, these synthetic processes being rendered possible through the presence of energy thereby made available. A large part of the plant nitrogen and the additional inorganic nitrogen are thus changed into organic nitrogenous compounds of microbial origin. As a result of this, the lowmoor peat is found to contain more nitrogen than the plants from which the peat has originated.

On the other hand, in the case of the sphagnum plants, the celluloses and hemicelluloses are not readily acted upon, under the anaerobic conditions prevailing in the bog, by microörganisms and hence offer only limited amounts of available energy for the building up of the microbial cell substance and the numerous microbial activities. Largely as a result of this, a part of the nitrogen liberated in the process of decomposition of the nitrogenous complexes of the dead sphagnum is thus left unassimilated by the microörganisms, and can be used by the growing plants. This accounts largely for the fact that the sphagnum peat may have less nitrogen than the living sphagnum plants.

The high cellulose and hemicellulose content of sphagnum peats serves as a further confirmation of these observations.

The same process of reasoning can be applied in an attempt to offer an explanation for the results of Kupreenko and Logvinova (24) who found that sphagnum peat contains a large quantity of its nitrogen (usually 11 to 14 per cent, and frequently as much as 19 to 28 per cent, of the total nitrogen) in the form of adsorbed ammonia, whereas lowmoor peat contains only traces of nitrogen in this form. When a comparison was made of the availability of nitrogen in the two forms of peat, using vegetation experiments, the sphagnum peat proved to be, during the first year growth of the plants, a much better

TABLE 5

Influence of depth of a lowmoor peat upon its decomposition at a constant moisture and air supply

100-gm. portions of moist peat incubated at 25–28°C. for 50 days

DEPTH OF SAMPLE	MOISTURE CONTENT	TOTAL CO_2 LIBERATED AS CARBON	AMMONIA FORMED	NITRATE FORMED	TOTAL NITROGEN LIBERATED
cm.	*per cent*	*mgm.*	*mgm. of N*	*mgm. of N*	*mgm. of N*
Surface	61.1	98.7	0.40	8.10	8.50
60	87.5	118.2	3.78	0.60	4.38
120	83.6	36.4	1.98	0.30	2.28
165	64.8	63.2	1.60	0	1.60

TABLE 6

Decomposition of sterile lowmoor peat (20 gm. dry matter), by different microörganisms, in 28 days

INOCULUM	CO_2 LIBERATED	AMMONIA FORMED	NITRATE FORMED	TOTAL NITROGEN LIBERATED	RATIO OF C/N LIBERATED
	mgm. of C	*mgm. of N*	*mgm. of N*	*mgm.*	
Soil suspension	68.7	5.8	3.8	9.6	7.16
Trichoderma	88.4	13.3	0.8	14.1	6.13
Actinomyces	87.7	11.6	1.8	13.4	6.54

source of nitrogen than the lowmoor peat. This phenomenon was naturally explained by the high content of adsorbed ammonia in the sphagnum peat, but no explanation was suggested for the origin of this ammonia. A study of the process involved in the decomposition of the two types of peat material would lead us to expect this very phenomenon to take place.

A comparison of the nitrate formation from lowmoor peats and from highmoor peat properly limed also brought out the fact (24) that, although the former contains much more nitrogen than the latter, this nitrogen is much more inert. Only 2.6 per cent of the total nitrogen was changed to nitrate in the case of a lowmoor peat composted for a period of five months, and 5 per cent in nine months; in the case of the highmoor peat, 9 per cent of the total nitrogen was changed to nitrate within two months, in the presence of enough CaO to bring the reaction to pH 6.5.

To measure the decomposition processes taking place in lowmoor peats, 100-gm. portions of fresh, moist Newton peat taken at four different depths were introduced into a series of flasks, placed in the incubator at 25–28°C., and connected with the aeration apparatus. Table 5 shows the rapidity of decom-

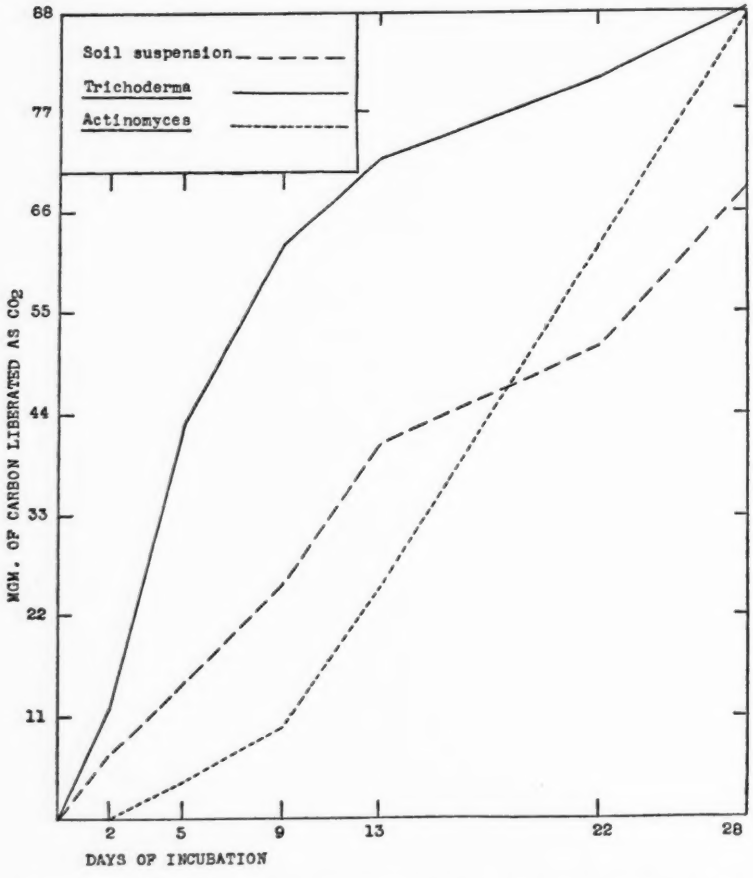

FIG. 1. COURSE OF DECOMPOSITION OF STERILE LOWMOOR PEAT BY A SOIL SUSPENSION AND BY PURE CULTURES OF MICROÖRGANISMS, AS MEASURED BY THE LIBERATION OF CO_2

position of 100-gm. portions of moist peat taken from different depths, as shown by the amount of carbon liberated as CO_2 and of nitrogen liberated as ammonia and nitrate, in a period of 50 days. The results show that in general the surface layers decompose with the greatest rapidity.

To determine the effect of the microbiological population upon the decomposition of peat, 70-gm. portions of Newton peat containing 20 gm. of dry matter were placed in flasks and sterilized at 15 pounds pressure for two hours. The flasks were then inoculated with a soil suspension or with pure cultures of the fungus *Trichoderma* or a typical soil *Actinomyces*. The amounts of CO_2 produced and of nitrogen liberated in 30 days are recorded in table 6, and the course of decomposition of the sterile peat by the two pure cultures and the soil suspension is given in figure 1. These results show that pure cultures of microörganisms, such as *Trichoderma* or *Actinomyces*, are capable of decomposing peat just as actively as and even more so than a mixed soil suspension. This is true in the case both of CO_2 production and of liberation of available nitrogen. It is interesting to note that nitrates were formed in

TABLE 7

Influence of inoculation, addition of inorganic nutrients and organic materials upon the decomposition of fresh lowmoor peat material

TREATMENT	CO_2 GIVEN OFF, MILLIGRAMS OF CARBON						NH_3–N	NO_3–N	TOTAL N LIBERATED
	Days of incubation								
	6	10	15	24	33	39			
							mgm.	*mgm.*	*mgm.*
Uninoculated	20.4	33.9	49.3	68.4	89.5	100.2	0.45	7.45	7.90
Suspension of fertile soil	22.7	36.5	54.4	73.7	95.1	106.8	1.17	6.72	7.91
Suspension of fresh cow manure	23.6	38.3	56.8	76.9	100.8	116.7	0.97	8.91	9.88
Mixture of *Actinomyces*	20.8	34.3	52.6	72.7	94.3	106.6	1.70	8.71	10.41
Green *Trichoderma*	21.8	36.3	53.4	73.5	96.3	108.3	2.67	7.77	10.44
400 mgm. $(NH_4)_2SO_4$ + 100 mgm. K_2HPO_4	26.7	43.9	64.0	83.6	103.6	115.7	73.81	6.58	.....
Cellulose, 2 gm	37.4	100.4	161.9	218.1	270.4	308.8	0.40	0.85	1.25
Rye straw, 2 gm	63.1	129.4	192.5	256.7	321.5	364.6	0.34	0.48	0.82

appreciable amounts only when the soil suspension was used as an inoculum. In the case of the *Trichoderma* and *Actinomyces*, all the nitrogen liberated in the decomposition of peat was in the form of ammonia. The small amounts of nitrate found in these cultures were largely present originally in the peat.

To throw further light upon the influence of inoculation as well as the addition of available salts and undecomposed organic matter upon the decomposition processes in natural fresh peat, another experiment was carried out. In a series of flasks were placed 100-gm. portions of fresh Newton peat containing 28 gm. of dry matter. These were variously inoculated. Some flasks received the addition of ammonium sulfate (80 mgm. of nitrogen) and dipotassium phosphate (100 mgm.). Others received 2-gm. portions of cellulose or rye straw. The flasks were placed in the incubator, connected with the aeration apparatus, and incubated for 39 days. At the end of the in-

cubation period, the amounts of ammonia and nitrate as well as the numbers of bacteria (by the plate method) were determined (table 7).

The uninoculated peat was found to contain 7,500,000 cells of microörganisms, developing on the plate, per gram, of which 80 per cent were actinomyces. There were also found 80,000 fungi per gram of peat. Inoculation with manure brought about an increase in the number of bacteria (probably due to their actual introduction with the manure) to 11,000,000 per gram, of which 65 per cent were actinomyces. The addition of a suspension of fertile soil did not modify to any considerable extent the bacterial and actinomyces population in the peat, but brought about a certain modification in the fungous population. The addition of nutrients increased somewhat the numbers of bacteria and reduced the numbers of fungi.

The fact that the addition of available nitrogen, phosphorus, and potassium did not bring about any appreciable increase in the evolution of CO_2 points definitely to the fact that nitrogen is not a limiting factor in the activities of microörganisms in peat but that the available carbon compounds are. When available energy, in the form of cellulose or rye straw, is added to the peat, rapid decomposition sets in, as indicated by the rapid increase in the evolution of CO_2. The addition of cellulose in the form of filter paper brought about a rapid increase in the numbers of fungi (species of *Cephalosporium*, *Fusarium*, *Trichoderma*, *Humicola*, *Penicillium*, but no Mucorales). When rye straw was added, the Mucorales, in addition to other fungi, developed quite abundantly. Various cellulose-decomposing bacteria, including *Spirochaeta cytophaga*, a very common form, developed in great abundance as a result of the addition both of cellulose and of straw. These results lead us to conclude that lowmoor peat is abundantly supplied with all microörganisms required for its rapid decomposition and for the liberation of its nitrogen in the form of nitrate. The organic complexes in the peat are very resistant to decomposition, as indicated by the fact that when only 2 gm. of cellulosic material is added to 100 gm. of peat containing 28 gm. of dry material there was three times as much carbon dioxide produced. This points to the comparative rapidity of decomposition of filter paper and straw, on the one hand, and to the slow decomposition of the organic complexes in peat, on the other. When cellulose and straw were added, there was practically no nitrogen left in the peat mixture, either as ammonia or as nitrate. This is because of the reassimilation of the available nitrogen by the microörganisms which use the cellulose and the straw as sources of energy.

To compare the results of the rapidity of peat decomposition with the decomposition of some of the plant materials which contribute to the formation of the peat, the stems (and leaves) and the roots or rhizomes of *Carex* and of *Cladium* (saw-grass) have been submitted to decomposition. A moisture content of 200 per cent, a nutrient solution containing ammonium phosphate and potassium chloride, and a soil suspension for inoculation were used. Twenty-gram portions of the dry material of *Carex* were placed in flasks and

40 cc. of water added. In the case of the *Cladium*, only 2-gm. portions were added to 100 gm. of quartz sand containing 20 cc. of water and the nutrient solution. The results of the evolution of CO_2 from the decomposition of these materials are given in figure 2.

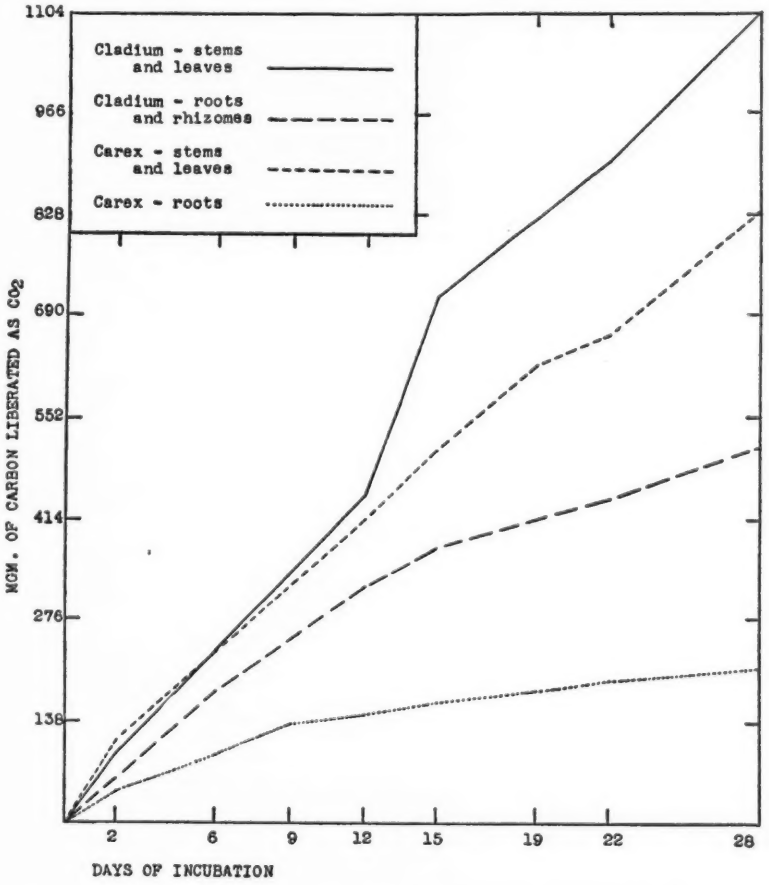

FIG. 2. DECOMPOSITION OF STEMS (AND LEAVES) AND ROOTS (AND RHIZOMES) OF CAREX (20 GM. OF DRY MATTER) AND CLADIUM (2 GM. OF DRY MATTER IN SAND CULTURES) PLANTS

In both cases, the stems and leaves decomposed much more rapidly than the roots and rhizomes. An analysis of these materials (44) shows that the stems and leaves are considerably richer in celluloses and pentosans than the roots, whereas the latter are much richer in lignins, which accounts for the difference

in the rapidity of decomposition. In the case of the *Cladium* stems, 5.2 mgm. of ammonia nitrogen was consumed from the inorganic solution, whereas in the case of the *Cladium* roots only 0.4 mgm. of ammonia nitrogen was used, because of less decomposition of the roots and greater protein content.

To determine the rapidity of decomposition of highmoor or sphagnum peat, two horizons were used: a light brown layer of younger sphagnum and a dark brown layer of older sphagnum peat of an Oldenburg profile from Germany, described elsewhere (41). Twenty-gram portions of dry peat and 40-cc. portions of tap water were introduced into a series of flasks; various salts were then added and the peat was incubated for 24 days. The amount of CO_2 produced was used as an index of the rapidity of decomposition of the peat.

Both the younger and the older sphagnum peats decomposed (table 8) less rapidly than the lowmoor peats. The older sphagnum decomposed somewhat

TABLE 8

Decomposition of 20 gm. portions of younger and older sphagnum peat from an Oldenburg peat profile

TREATMENT OF PEAT	CO_2 LIBERATED, MGM. OF CARBON					
	Young sphagnum*			Old sphagnum†		
	Days of incubation					
	5	10	24	5	10	24
Untreated	8.6	16.4	29.4	8.7	18.4	33.6
500 mgm. $(NH_4)_2HPO_4$ + 100 mgm. K_2HPO_4	14.1	22.8	38.2	12.2	20.8	36.7
1 gm. $CaCO_3$	41.3	56.6	88.3	53.5	72.3	108.8
50 mgm. $CuSO_4$	8.6	17.9	36.1	10.8	19.4	35.2

* pH of peat 4.1, nitrogen content, 0.83 per cent.

† pH of peat 4.4, nitrogen content 0.99 per cent.

more rapidly than the younger; it was less influenced by the addition of available nitrogen salt, but more so by the addition of $CaCO_3$. The large amount of CO_2 liberated in the presence of $CaCO_3$ is due chiefly to the chemical interaction between the organic acid reacting complexes and the carbonate. The addition of $CuSO_4$ seemed to have no effect upon the rapidity of decomposition of both layers of peat.

To establish the rapidity of decomposition, a sphagnum peat from Maine (Cherryfield) described elsewhere (43), recently taken from the bog, with and without additional inorganic salts and carbonate, was used. Sufficient air-dry peat was used to give 25 gm. of dry material; enough water was added to bring it to 300 per cent moisture. The peat was placed in aeration flasks, inoculated with fresh soil suspension and incubated at 25–27°C. for 19 days. The amount of CO_2 liberated was determined at frequent intervals. At the end of the incubation period, the amounts of nitrogen liberated in an inorganic

form (ammonia + nitrate) were determined. Only traces of nitrates were produced in all flasks. The results are given in table 9.

Here as well, the addition of ammonium sulfate and dipotassium phosphate had no stimulating effect upon the processes of decomposition. This is primarily because in the sphagnum peat, the nitrogenous complexes decompose rapidly, whereas the carbohydrates, which are quite abundant in this type of peat (44), are quite resistant to decomposition. The ratio between the carbon liberated as CO_2 and the nitrogen liberated as ammonia and nitrate is narrow in the case of the lowmoor and sedimentary peats, ranging from 12:1 to 13:1 in the case of the Newton peat, to 3:1 for the saw-grass peat in Florida, and only about 1.3:1 for the sedimentary peat. In the case of the highmoor or sphagnum peat, however, this ratio is about 20:1. The only exception in respect to this ratio is found in the lowmoor peats taken from lower depths. The results presented in table 5 show that the deeper the layer of peat in the bog, the wider is the ratio between the CO_2 and the nitrogen liberated.

TABLE 9

Decomposition of sphagnum peat under different treatments

TREATMENT	CO_2 LIBERATED	NH_3-N and NO_3-N
	mgm. C	*mgm. N*
Untreated	94.4	4.65
100 mgm. $(NH_4)_2SO_4$ + 100 mgm. K_2HPO_4	86.6	18.95
0.5 gm. $CaCO_3$	125.1	4.55
1.0 gm. $CaCO_3$	146.3	4.25
1.0 gm. $CaCO_3$ + 100 mgm. K_2HPO_4	135.7	4.05
1.0 gm. $CaCO_3$ + 100 mgm. $(NH_4)_2SO_4$ + 100 mgm. K_2SO_4	134.1	18.30

To determine the influence of moisture upon the decomposition of peat, a part of the lowmoor peat used in the previous investigations was air-dried, then adjusted, by the addition of water, to various moisture contents. The drying of the peat made it in some manner less readily available to the action of microörganisms and less capable of absorbing moisture. Whereas natural lowmoor peat may contain as much as 92 per cent moisture and 8 per cent dry matter or over 1000 per cent moisture, the dried material could not absorb more than 400 per cent moisture and the excess water remained in a free state. It is possible that the drying results in a change in the physical condition of the hemicelluloses into horny-like substances; these may be irreversible in nature and may exert a protective effect upon the decomposition of the various complexes in the peat. Possibly the removal of this hemicellulose from the peat by the HCl treatment, as shown in the following experiments was one of the factors favoring its more rapid decomposition.

The influence of moisture content and addition of inorganic nitrogen upon the decomposition of peat is shown in table 10. Ammonia and nitrate were determined at the end of the incubation period, namely, after 20 days. The

addition of inorganic nitrogen exerted again only a minor influence upon the rapidity of decomposition, as shown by the evolution of CO_2. The undried peat decomposed most rapidly. The air-drying of the peat had an injurious effect upon the rapidity of its decomposition and also upon the nitrifying bacteria. Whereas in the fresh peat the nitrogen liberated is rapidly changed to nitrate, in the dried peats most of the nitrogen liberated remained as ammonia. The lowest amounts of CO_2 were given off with the highest moisture content. In the presence of an excess of water, conditions became more and more anaerobic, favoring the activities of anaerobic bacteria. The lack of depression in the liberation of available nitrogen shows that even under partial

TABLE 10

Influence of moisture and additional nitrogen upon microbiological activities in lowmoor peat

On the basis of 20 gm. of dry peat

MOISTURE CONTENT	NITROGEN ADDED*	CO_2 GIVEN OFF, MILLIGRAMS OF CARBON				NH_3-N	NO_3-N	TOTAL NITROGEN
		2 days	8 days	12 days	20 days			
per cent						*mgm.*	*mgm.*	*mgm.*
Natural peat†	−	14.8	34.5	46.8	64.3	0.3	11.3	11.6
Natural peat†	+	16.8	37.9	50.9	68.7	0.3	22.2	22.5
200‡	−	8.3	23.1	35.0	54.4	7.9	1.5	9.4
200	+	9.0	24.3	36.1	54.5	17.5	1.7	19.2
300	−	6.6	20.6	31.0	48.9	8.2	0.8	9.0
300	+	8.2	23.8	34.7	51.6	18.4	1.0	19.4
450	−	3.4	13.9	20.2	32.2	9.1	0.9	10.0
450	+	3.4	14.9	23.3	36.4	20.6	0.9	21.5

* 10 mgm. of nitrogen added in the form of ammonium sulfate.

† Fresh peat containing 67 per cent moisture.

‡ Peat previously air-dried, then adjusted to 200, 300, and 450 per cent moisture.

anaerobic conditions the nitrogenous complexes are readily decomposed. The higher the moisture content of the peat the narrower was the C:N ratio or the ratio between the carbon liberated as CO_2 and the nitrogen as ammonia.

Influence of peat treatment upon its decomposition by microörganisms

Wollny (47) found that on removing the waxes from peat with ether, the rapidity of its decomposition doubled. To throw further light upon this subject, a quantity of the lowmoor peat profile from Newton was divided into three portions. One portion was left untreated. The second portion was treated with toluene for 48 hours and the toluene allowed to evaporate. The third portion was heated with a solution of 2 per cent HCl, for one hour at 15 pounds pressure in the autoclave; the peat was then filtered and washed with tap water until practically free from acid; the remaining acidity was neutralized by the addition of a small amount of CaO.

Enough peat of each of the three preparations was introduced into a series of flasks to give 20 gm. of dry material; the moisture was then adjusted to 200 per cent. All flasks were inoculated with a suspension of good garden soil and incubated for 20 days at 25°C.

The results (table 11) show that treatment of peat with toluene greatly favored its decomposition, as shown by the evolution of carbon dioxide, the liberation of nitrogen in an available form, and the development of bacteria. The numbers of fungi diminished. The nitrifying organisms were injured somewhat, as a result of which most of the nitrogen was left in the form of ammonia. Treatment of the peat with dilute acid at a high temperature resulted in the removal of a large part of the protein and of the hemicellulose. Nevertheless the remaining material decomposed much more rapidly than the untreated peat, showing that either some protective substance or some injurious substance was removed by the treatment. It is interesting to note the number

TABLE 11

Influence of treatment of lowmoor peat upon the growth and activities of microörganisms

On basis of 20 gm. of dry peat (20 days)

TREATMENT OF PEAT	UNTREATED	TOLUENE TREATED	HEATED WITH 2 PER CENT HCl
Mgm. of carbon liberated as CO_2	64.3	95.3	69.0
Mgm. of nitrogen liberated as ammonia	0.3	21.1	17.1
Mgm. of nitrogen accumulated as nitrate	11.3	5.9	1.1
Total nitrogen liberated, mgm.	11.6	27.0	18.2
Numbers of bacteria in 1 gm. of moist peat	8,300,000	46,000,000	7,000,000
Numbers of fungi in 1 gm. of moist peat	160,000	80,000	10,000,000

of fungi growing on the HCl-treated peat in comparison with the untreated and the toluene treated. The nitrifying bacteria were completely depressed and have not recovered as a result of inoculation, hence the nitrogen liberated was found entirely in the form of ammonia. Further experiments dealing with the influence of treatment of peat with ether, toluene, and dilute acid confirmed these observations.

Different treatments seem to influence differently the decomposition of the various organic complexes in the peat, as shown in table 12. The greatest effect upon the decomposition of the peat constituents was exerted by the ether treatment, as shown by the evolution of carbon dioxide. However, the lowest amount of nitrogen was liberated as a result of this treatment. This is no doubt because the non-nitrogenous complexes have been made, as a result of treatment with ether, more readily decomposable. Possibly some of the nitrogen might have been, as a result of that, reassimilated by the micro-organisms, because of the very extensive development of both fungi and bacteria. Here as well, treatment with toluene and hot hydrochloric acid

resulted in an increase in the decomposition of the peat, as shown by the liberation of CO_2 and ammonia.

The fact that treatment with ether favors the decomposition of the non-nitrogenous complexes in peat is shown in table 13. Although the decomposition of highmoor peat was stimulated considerably on treatment with ether, as shown by the evolution of CO_2, there was a reduction of the amount of available nitrogen liberated as ammonia, pointing to a reassimilation of a part

TABLE 12

Influence of treatment of lowmoor peat upon the growth and activities of microörganisms

On basis of 20 gm. of dry peat (22 days)

TREATMENT OF PEAT	UNTREATED	ETHER TREATED	TOLUENE TREATED	HEATED WITH 2 PER CENT HCl
Mgm. of carbon liberated as CO_2	36.1	204.7	62.1	146.0
Mgm. of nitrogen liberated as ammonia	0.3	7.9	9.0	14.9
Mgm. of nitrogen liberated as nitrate	11.8	0.9	6.4	1.1
Total nitrogen liberated	12.1	8.8	15.4	16.0
Numbers of bacteria and actinomyces in 1 gm. of moist peat	21,000,000	97,000,000	25,000,000	105,000,000
Per cent of actinomyces	50	10	5	5
Numbers of fungi in 1 gm. of moist peat	235,000	7,075,000	51,000	359,000

TABLE 13

Influence of treatment of highmoor peat (Maine) upon its decomposition by microörganisms

On basis of 20 gm. of dry peat (21 days)

TREATMENT OF PEAT	UNTREATED	1 GM. $CaCO_3$ ADDED	50 MGM. $(NH_4)_2HPO_4$ ADDED	TREATED WITH ETHER
Mgm. of carbon, liberated as CO_2	61.40	98.4	61.30	97.9
Mgm. of nitrogen, as ammonia	5.95	4.9	13.25	3.0

of the nitrogen by the microörganisms decomposing the carbonaceous complexes. The addition of available nitrogen had no effect upon the rapidity of decomposition of sphagnum peat.

The marked increase in the decomposition of peat following treatment with ether or with toluene, as measured by the evolution of CO_2 in the process of aerobic decomposition, points to some interesting generalizations. The removal of the fats and waxes, or ether-soluble substances, from lowmoor peat, brought about an evolution of CO_2 which was about five times as great as that of the untreated peat. The treatment of peat with toluene, even without the removal of the toluene extract but merely allowing the solvent to evaporate, more than doubled the rate of peat decomposition.

We can hardly explain this by the destruction of certain groups of microorganisms, such as protozoa, or by a stimulating effect of the disinfectant, but rather by a change produced in the chemical condition of the organic complexes of the peat. It was shown elsewhere that the removal of the ether-soluble material from fresh plant material, such as straw, favors its more rapid decomposition. We may expect similar results, probably even more marked, from the treatment of peat or of soil organic matter.

SUMMARY

1. Results of investigations on the occurrence and activities of microorganisms in different peat bogs are reported.

2. These results prove conclusively that microörganisms play a most important rôle in the formation of peat from the plant remains.

3. In lowmoor peat bogs the numbers of aerobic bacteria diminish rapidly with depth whereas the numbers of anaerobic bacteria increase rapidly with depth.

4. Fungi, aerobic cellulose-decomposing bacteria, and nitrifying bacteria are found in lowmoor peat bogs at or just below the surface of the bog, then diminish rapidly and disappear completely at a depth of 75 to 90 cm. Actinomyces are abundant at the surface of the lowmoor peat but they also diminish with depth but not so rapidly as the fungi; they disappear completely at a depth of 120–150 cm.

5. Acid sphagnum peat bogs contain an abundant flora of acid-resistant bacteria capable of growing in media of pH 4.0. In undrained sphagnum peat bogs, the numbers of bacteria, largely anaerobic forms, increase with depth, so that at a depth of 570 cm. there were found more bacteria growing on synthetic agar media than in the surface layers of the bog.

6. The rate of decomposition of peat, as shown by the evolution of CO_2, is much slower than that of fresh plant residues.

7. With an increase in depth of peat there is a widening of the ratio (C/N) between the carbon liberated as CO_2 and the nitrogen liberated as ammonia and nitrate. The deeper the lowmoor peat the less active is its nitrifying capacity.

8. Certain pure cultures of fungi and actinomyces can decompose sterilized lowmoor peat as fast as the total soil population.

9. The addition of inorganic nitrogen salt and phosphates had practically no effect upon the rapidity of decomposition of lowmoor and highmoor peats, because available energy and not nitrogen is the limiting factor in the decomposition of peat material.

10. The ratio between the CO_2 and nitrogen liberated (C/N) in the course of peat decomposition is wider in the case of the highmoor sphagnum peats than in lowmoor peats.

11. Treatment of peat with ether, toluene, and dilute hydrochloric acid

followed by the removal of the reagent leads to a very marked increase in the rapidity of the peat decomposition.

12. The action of ether and toluene is not due so much to any change in balance of the microbial population of peat as to the removal of waxy substances rendering the peat more readily available for the action of microörganisms.

13. Different treatments differ markedly in the nature of their action upon peat, because different organic complexes in the peat are affected by each treatment.

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